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The Journal of Laboratory and Clinical Medicine

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CLINICAL AND EXPERIMENTAL

THE BLOOD PIGMENTS*

THE PROPERTIES AND QUANTITATIVE DETERMINATION WITH SPECIAL REFERENCE TO THE SPECTROPHOTOMETRIC METHODS

H. O. MICHEL, PH.D., AND J. S. HARRIS, M.D.
DURHAM, N. C.

WITH the increasing prevalence of aniline-containing drugs and poisons in the past few years, a number of reports concerning the presence of abnormal pigments in the blood have appeared. The constitution of the hemoglobin derivatives has been but recently clarified, and the nomenclature has not as yet been standardized. Methods for the quantitative determination of methemoglobin and sulfhemoglobin have not been adequately reviewed. At present, they are often not specific for the pigment in question, and are sufficiently cumbersome to limit clinical studies to small series of cases. We have reviewed (in this study) the interrelationships and characteristics of blood pigments and presented a critical discussion of existing methods, together with rapid, simple methods, for the spectrophotometric determination of methemoglobin and sulfhemoglobin.

NATURE OF THE BLOOD PIGMENTS[1-3]

The prosthetic group of the hemoglobin compounds is an iron protoporphyrin complex termed *heme* by Anson and Mirsky.† The reduced, or ferroheme, compound may be reversibly oxidized at the iron atom to ferriheme

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†Nomenclature of the Blood Pigments. Pauling and Coryell[2] have recently advanced certain improvements in the nomenclature. The following table is added to simplify the relationships. The terms of Pauling and Coryell are italicized while those of Anson and Mirsky[1] are in parentheses.

Heme: A generic term for any iron porphyrin complex.

(hematin).[4] The latter combines with acids to form ferriheme salts (e.g., ferriheme chloride) or with alkalies to form ferriheme hydroxide (alkaline hematin).[5] Ferroheme may combine with certain nitrogenous substances (e.g., proteins, pyridine, cyanides) to form ferrohemochromogens,[6] while ferriheme forms a corresponding series of ferrihemochromogens (parahematins).[5] The combination of ferriheme and native globin has been performed in vitro and results in ferrihemoglobin (methemoglobin).[7] Ferroheme and native globin form ferrohemoglobin (hemoglobin). The oxidation of hemoglobin which results in ferrihemoglobin (methemoglobin) is to be contrasted with the oxygenation of hemoglobin which results in the formation of the addition complex, oxyferrohemoglobin (oxyhemoglobin).

Sulfhemoglobin has never been prepared in a pure state and its constitution is not known.[9] Upon disrupting the molecule, one obtains the same heme and porphyrin as in the case of hemoglobin.[10] Alkaline denaturation experiments show that there is no major change in the globin portion of the molecule.[11] The formation of sulfhemoglobin apparently involves the introduction of one atom of relatively labile sulfur into the hemoglobin structure and also a change in the mode of linkage of the globin to the heme. At the present time, there is no way of converting sulfhemoglobin into either methemoglobin or hemoglobin without first splitting the molecule into its constituent groups.

The relationship between the blood and bile pigments is best summarized by a consideration of the recent work of Lemberg[12] and of Barkan and Sehales.[13] Under certain experimental conditions, the partial oxidation of hemoglobin or hemochromogen leads to an opening of the porphyrin structure at the alpha methene bridge without a loss of the central iron atom. To this type of compound Barkan has applied the term pseudohemoglobin, while Lemberg calls them verdohemochromogens. A characteristic of this group is the ease with which the iron may be split off by mild acids to give rise to the bile pigment, biliverdin. Pseudohemoglobin may furnish the link between hemoglobin and the bile pigments. Barkan has suggested that the 4 to 6 per cent of labile iron in the red blood cells[14] may be due, in part, to the presence of pseudohemoglobin.[15]

CHEMICAL PROPERTIES

Hemoglobin combines reversibly with oxygen, carbon monoxide, and nitrous oxide.[16] Through the action of strong acids or bases upon hemoglobin, the cor-

---

Ferroheme (reduced heme): Ferrous porphyrin complex. Unless specified, the porphyrin is protoporphyrin.

Ferriheme (oxidized heme): Ferric porphyrin complex.

Ferriheme chloride (hemin): Teichmann's crystals. Ferriheme plus chloride ions. The general term for these salts is acid hematin.

Ferriheme hydroxide (alkaline hematin): Ferriheme plus hydroxyl ions.

Ferrohemochromogen (hemochromogen): Complex of ferroheme and one or two nitrogenous substances, such as pyridine, nicotine, ammonia, etc.

Ferrihemochromogen (parahematin): Also called kathematin. Complex of ferriheme and nitrogenous substances.

Hemoglobin: A generic term denoting the complex or an iron porphyrin and native globin.

Ferrohemoglobin (reduced hemoglobin or hemoglobin): Complex of ferroheme and native globin.

Oxyhemoglobin: The complex of ferrohemoglobin and oxygen.

Globin ferrohemochromogen: The complex of ferroheme and denatured globin.

Ferrihemoglobin (methemoglobin): The complex of ferriheme and native globin.

Globin ferrihemochromogen (globin parahematin or kathemoglobin): The complex of ferriheme and denatured globin.

A system for naming the hemochromogen derivatives is given by Drabkin.[8]

responding ferrihemes are formed. Oxidation with hydrogen peroxide in the presence of large amounts of cyanide or coupled oxidation with ascorbic acid results in the formation of pseudohemoglobin.[13] Strong oxidation with a variety of agents changes the valence of the iron to form methemoglobin. Hemoglobin and oxyhemoglobin do not react with cyanides, fluorides, or azides.

Methemoglobin forms a reversible redox equilibrium with hemoglobin. The E_o at pH 7 is + 150 millivolts.[17] Ozone, iodine, chlorates, ferricyanide, hydroxylamine, hydroquinone, p-amino phenol, formaldehyde, hydrogen peroxide, pyrogallol, permanganate, and certain bacteria (*Streptococcus viridans*, cholera vibrio, pneumococcus, Gärtner's bacilli, and certain nitrosobacilli) can convert hemoglobin to methemoglobin.[18] Methemoglobin forms spontaneously when solutions of purified hemoglobin are exposed to oxygen. The free energy of this reaction is sufficiently great to preclude the spontaneous occurrence of the reverse reaction.[19] However, in vivo or in drawn whole blood, the reverse reaction may take place through the action of reducing substances (glucose, unsaturated fatty acids) which are present.[3] Methemoglobin is reduced to hemoglobin by the action of sodium hydrosulfite, ammonium sulfide, sodium anthrahydroquinone disulfonate, titanous tartrate, and Stokes' reagent. Methemoglobin combines reversibly with cyanides (formation of cyanmethemoglobin),[20] sulfides (sulfmethemoglobin),[21] peroxides,[22] fluorides,[23] and azides.[24] Like the other ferriheme compounds, it may exist as the base (alkaline methemoglobin) or as the salt (acid methemoglobin).[25] It does not combine with oxygen or carbon monoxide.

Sulfhemoglobin is formed by the reaction of hemoglobin with soluble inorganic sulfides and hydrogen peroxide.[11] The reaction of oxyhemoglobin with sulfides is slow but is considerably accelerated by certain reducing agents (phenylhydrazine, hydrazine, hydroxylamine, naphthylamine, phenylenediamine, isatine, paraminophenol, and particularly hydrosulfite).[27] Sulfhemoglobin exists in the oxidized or reduced form.[21] As ordinarily prepared through the action of sulfide and a reducing agent on oxyhemoglobin, the reduced form is obtained. Oxidized sulfhemoglobin may be prepared by the action of ferricyanide on the reduced form. Reduced sulfhemoglobin reacts with carbon monoxide,[27a] but not with oxygen, cyanide, sulfides, ammonia, azide, hydrosulfite, Stokes' reagent, alkalies, or fluorides.[26] Hemochromogen is formed upon the addition of mercuric chloride or strong bases followed by reduction with hydrosulfite.[28]

Hematin is formed through the action of strong acids or bases upon hemoglobin. As has been mentioned previously, it may exist as the base or salt. Hematins combine with cyanides to form cyanhematin and with other nitrogenous compounds to form parahematins.[5] Upon reduction, hematins form unstable ferrohemes which rapidly revert to hematin in the presence of oxygen. Both hematin and cyanhematin form ferrohemochromogens upon the addition of ammonium sulfide.[29] This behavior is to be contrasted with that of methemoglobin or cyanmethemoglobin which slowly revert to reduced hemoglobin under the same conditions. Ferroheme and ferrohemochromogen, particularly in alkaline solutions, combine with carbon monoxide but not with oxygen.[30] Hematin has been reported in the blood plasma and urine of patients with malaria, pernicious anemia, intravascular hemolysis, and sepsis.[31]

A pigment, known as pseudomethemoglobin, may appear in the plasma of patients with blackwater fever or massive intravascular hemolysis.[32, 33] This pigment is identified by the presence of an absorption band between 622 and 624 mμ, by its reaction with sodium hydrosulfite, and by its failure to react with Stokes' reagent or 10 per cent ammonium sulfide. The composition is not known. Fairley and Bromfield[32] have suggested that pseudomethemoglobin is a combination of hematin with unknown nitrogenous constituent of the plasma, probably serum albumin. The pigment has not been found in red corpuscles.

Verdohemochromogen has not been demonstrated spectroscopically in the blood stream. Its existence is assumed because of the presence of the easily split off iron of the red blood cells.

SPECTROPHOTOMETRIC PROPERTIES

Investigations concerning the nature of the blood pigments have depended largely upon their color, because of their relatively sharp absorption bands and the ease of spectroscopic examination. It is to be emphasized that, at the present time, the identification of the various blood pigments still depends mainly upon their spectral properties. For example, the characterization of sulfhemoglobin and pseudomethemoglobin is based solely upon the position of their absorption bands and the behavior of these bands under chemical manipulation.

(a) *Theoretical.*[34, 35]—The change in intensity of light, as it passes through an absorbing medium, is given by the following equation:

$$-\frac{dI}{dl} = KI \qquad (1)$$

Upon integrating and changing to logarithms to the base 10, we obtain,

$$-\log \frac{I_l}{I_o} = El \qquad (2)$$

where

I_o = intensity of incident light.
I_l = intensity of light at depth of solution 1.
E and K are constants.

For monochromatic light the absorption is generally proportional to the number of molecules in the path of the light. The relationship is given by the Beer-Lambert[36] law which has been verified for dilute solutions of the blood pigments.[37]

$$-\log \frac{I_l}{I_o} = E\ cl \qquad (3)$$

where

$$E = \frac{E}{c} \text{ and } c = \text{concentration of absorbing material.}$$

E is known as the extinction coefficient (transmissive index) and is equal to $-\log \frac{I_l}{I_o}$ (optical density or extinction) when the depth of the solution is unity. E is the specific extinction coefficient (specific transmissive index) and is equal to the optical density at unit depth and concentration. If $\frac{I_l}{I_o}$ equals $1/10$, then $E\ cl = 1$, and the physical meaning of the extinction coefficients becomes

clear. The specific extinction coefficient is the reciprocal of that concentration necessary to reduce the light to $\frac{1}{10}$ of its original intensity in a unit layer of solution.[38*]

Qualitative spectrophotometry depends upon the fact that each substance exhibits a characteristic relationship between extinction and wave length, i.e., a characteristic absorption spectrum. This relationship is generally expressed by the curve obtained when the extinction coefficients are plotted as ordinates against the wave length as abscissae.[39] However, when the concentration is varied or when different units of concentration are used, it is found that the curves for a particular substance cannot be superimposed. The curves will vary both in form and in height, since $E = E/C$. This effect of concentration in changing the form of the curve is canceled if one plots the logarithm of the extinction coefficient against the wave length.[34] Curves for the same substance at different concentrations can then be superimposed. They run parallel to each other at an interval equal to log c, since $\log E = \log E - \log c$.[40] By plotting the data on semilogarithm paper, one retains the simplicity of the first method and the advantages of the unchanging form of the second method ("typical absorption curves"). This type of curve becomes particularly important when the concentration of the substance is not known.

(b) *Absorption Spectra.*—The "typical absorption spectra" of some of the blood pigments and their derivatives are shown in Figs. 1 and 2. Oxyhemoglobin is easily distinguished by the characteristic bands at 540 and 575 mμ. Upon the addition of strong reducing agents (e.g., hydrosulfite) both bands disappear, to be replaced by the single strong band at 555 mμ of reduced hemoglobin. Addition of oxidizing agents (ferricyanide) in neutral or acid solutions causes the appearance of the alpha methemoglobin band at 630 to 634 mμ. This methemoglobin band disappears upon the addition of a few grains of cyanide, hydrosulfite, sulfide, azide, fluoride, or alkali. Sulfhemoglobin has a characteristic band at 618 to 620 mμ which is unchanged by the addition of the reagents mentioned above in the case of methemoglobin. The band disappears upon the addition of ferricyanide and is shifted from 5 to 7 mμ towards the violet when carbon monoxide is bubbled through the solution. The methemoglobin band, on the contrary, is not changed by the addition of carbon monoxide. Illuminating gas must be purified for this purpose since it may contain sulfides or reducing agents which will alter methemoglobin. Hematin in acid solution shows a strong alpha band at 660 mμ. In N/10 sodium hydroxide, the alpha band is less prominent and has its maximum at 610 mμ. When seen in serum, hematin generally shows a band at 610 to 625 mμ. The band disappears upon addition of cyanide. Cyanide and ammonium sulfide or sodium hydrosulfite disperse the band with the formation of a hemochromogen spectrum (sharp bands at approximately 560 and 530, the former band being the stronger).

(c) *Qualitative Spectroscopic Examination of the Blood.*—Freshly drawn, oxalated blood is placed in a small test tube and centrifuged. The plasma is

*Frequent use is made of the term *absorption constant* or *absorption ratio*, which is designated by A. The definition is given by the equation

$$c = A \times E \qquad (4)$$

A is the reciprocal of the specific extinction coefficient (usually in units of grams solute per cubic centimeter of solution).

removed and the cells are diluted with an equal quantity of water. Hemolysis is completed by the addition of a few milligrams of saponin. The mixture is stirred cautiously since shaking produces a foam which hinders the examination. Whole blood may be hemolyzed with saponin without the addition of water if one is not interested in the plasma pigments. Illumination is furnished by a small vertical beam from a shielded 100 watt frosted bulb. Through a low dispersion, direct vision, and hand spectroscope, various thicknesses of solution are examined by tilting the test tube.* A point will be found where only the red region of the spectrum is light enough to permit the detection of absorption bands. The 630 methemoglobin band appears to divide the red region at a point approximately 4/10 of the distance from the yellow end. The 620 sulfhemoglobin band is slightly nearer the alpha hemoglobin band. A practiced observer can detect this difference with the hand spectroscope even before the application of the usual confirmatory tests (disappearance of the methemoglobin band but stability of the sulfhemoglobin band on addition of a few drops of 1 per cent potassium cyanide,[44] dilute ammonia, or a few grains of sodium hydrosulfite). The band of sulfhemoglobin can be abolished by the addition of ferricyanide, but cyanide must be added to prevent the appearance of the methemoglobin absorption in that region. If hydrosulfite is now added, the sulfhemoglobin band at 620 returns.

The sensitivity of this test does not depend upon the absolute concentration of the abnormal pigment but on the relative intensities of the abnormal band and the diffuse absorption of oxyhemoglobin in that portion of the spectrum. Methemoglobin can be detected when it constitutes as little as 3 to 4 per cent of the total pigment (approximately 0.5 gm. per cent).[45] Since the sulfhemoglobin band is about three times as strong as that of methemoglobin, 1 per cent sulfhemoglobin in normal blood (0.1 gm. per cent) can be seen.[43a] The simultaneous occurrence of sulfhemoglobin and methemoglobin is indicated when the band in the red becomes lighter but does not disappear upon the addition of cyanide, and seems to be shifted toward the blue end of the spectrum.

Examination of the plasma can be carried out in similar fashion. Slight hemolysis will cause the appearance of the two banded oxyhemoglobin spectrum. Methemoglobin is detected as described above. Hematin produces a band at approximately 610 to 630 mμ. Upon the addition of cyanide, this band disappears. Upon the further addition of ammonium sulfide, a hemochromogen spectrum, with bands at 530 and 560, appears. This serves to distinguish clearly between methemoglobin and hematin, since methemoglobin reverts to reduced hemoglobin under these conditions. Pseudomethemoglobin shows a band at 622 which does not change on addition of Stokes' reagent or 10 per cent ammonium sulfide, but disappears upon the addition of sodium hydrosulfite.

*Greater elegance in this examination may be achieved through the use of easily constructed apparatus.[43] The use of a glass wedge simplifies the examination of various depths of solution.

QUANTITATIVE DETERMINATION OF THE BLOOD PIGMENTS

Methods for the quantitative determination of the blood pigments may be divided into three general types: gasometric, combined gasometric and colorimetric, and spectrophotometric.

(a) *Gasometric.*—Gasometric methods have been applied only to the determination of hemoglobin and methemoglobin. They are based upon the determination of the oxygen or carbon monoxide combining power of blood before and after the conversion of methemoglobin into hemoglobin through the action of reducing agents. The carbon monoxide capacity method was first suggested by Nicloux and Fontes[46] and elaborated by Van Slyke and his coworkers.[47] This method is given in detail by Peters and Van Slyke.[48] The oxygen capacity method was improved by Conant and Fieser.[49] The oxygen capacity is determined by the Van Slyke and Neill[50] method before and after reduction with sodium anthrahydroquinone B sulfonate[49] or titanous tartrate.[51]

The gasometric methods require experience and are tedious. The oxygen capacity method is specific for methemoglobin, but there is always the danger of the reoxidation of the laked hemoglobin to methemoglobin during the aeration with oxygen. This tends to make the final result too low, but the error is minimized by the use of titanous tartrate as the reducing agent. As Conant[51] has pointed out, the carbon monoxide methods are disturbed by the presence of other pigments. After reduction, both hematin and sulfhemoglobin may combine with carbon monoxide and thus appear in the result as methemoglobin. In both methods methemoglobin is calculated from the difference between two determinations, thus magnifying the errors. There are no gasometric methods for sulfhemoglobin or other pigments.

(b) *Combined Gasometric and Colorimetric Determinations.*—Active hemoglobin is determined by the carbon monoxide or oxygen combining power. Total pigment is determined by a variety of methods: colorimetric cyanmethemoglobin,[52, 53] acid hematin,[54] methemoglobin,[55] or total iron.[56] The difference between the active hemoglobin and the total pigment represents the methemoglobin or sulfhemoglobin.

These methods are not specific. If the oxygen capacity is used for the determination of the active hemoglobin and the total iron for total hemoglobin, the result will represent all other iron-containing pigments: methemoglobin, sulfhemoglobin, hematin, and some nonhemoglobin iron. In the case of the carbon monoxide methods, a variable amount of sulfhemoglobin may combine with the gas and be calculated as active hemoglobin. The difficulties of the colorimetric methods have been discussed by Schwentker.[57] They require fresh standards which are difficult to prepare and must be constantly rechecked. Turbidity,[58] hematin, or other colored substances interfere with the cyanmethemoglobin and methemoglobin methods. The iron analyses are more accurate but are more difficult to conduct and generally require preliminary ashing.

When both methemoglobin and sulfhemoglobin are present, the only combined method would require the determination of the difference in oxygen capacity before and after reduction with titanous tartrate as a measure of

methemoglobin followed by the determination of total pigment by the acid hematin or iron analytic methods to obtain the sulfhemoglobin concentration. Nonhemoglobin iron naturally interferes.

(c) *Spectrophotometric Methods.*[34, 35, 59-61*]—Quantitative spectrophotometry depends upon the equations previously derived, and the fact that the total extinction coefficient for a solution is an additive function of the separate extinction coefficients of its constituents in the absence of interaction.

$$E_t = E_1 + E_2 \qquad (5)$$

Utilizing these principles, the following spectrophotometric methods have been evolved:

(1) The extinction coefficient of a solute may be calculated by subtraction of the extinction of the remainder of the materials in solution from the total observed extinction. This operation is performed automatically if one places the solution containing the compound in question in one cup of the spectrophotometer and the solution without the compound in the other cup. The concentration of the solute may be readily determined from the observed extinction if the specific extinction coefficient is known.

$$\text{conc. (x)} = \text{E (observed)} \; / \; E_x \qquad (6)$$

(2) Method of Vierordt.[59, 62] The concentrations of two colored substances in an optically clear solution may be calculated from the total extinction coefficients of the solution at two wave lengths through the use of simultaneous equations.

Let

E_{am} and E_{bm} be the extinction coefficients of the solution at wave lengths a and b.

E_{ax}, E_{ay}, E_{bx}, and E_{by} be the known specific extinction coefficients of components x and y at wave lengths a and b.

X and Y be the concentrations of components x and y in the solution.

From 5 and 6 we obtain

$$E_{am} = E_{ax}\,X + E_{ay}\,Y \qquad (7)$$

$$E_{bm} = E_{bx}\,X + E_{by}\,Y \qquad (8)$$

Substituting and rearranging

$$X = \frac{E_{by}\,E_{am} - E_{bm}\,E_{ay}}{E_{by}\,E_{ax} - E_{ay}\,E_{bx}} \qquad (9)$$

$$Y = \frac{E_{ax}\,E_{bm} - E_{am}\,E_{bx}}{E_{by}\,E_{ax} - E_{ay}\,E_{bx}} \qquad (10)$$

These principles may be extended to solutions containing three or more solutes. Application and criticism of this method will be discussed with those of the following procedure.

---

*The chief drawback to spectrophotometric methods is the expense of the apparatus. Although we realize that these instruments are by no means in widespread clinical use, the principles involved have been explained fully since they form the basis of all colorimetric methods.

Recently photoelectric photometers have become increasingly popular because of their convenience, simplicity, and accuracy. All methods used in these instruments are based upon spectrophotometric principles, and the spectrophotometer is essential in the development of new applications. Conversely, all spectrophotometric methods are easily adapted to the photoelectric photometers if one has the choice of suitable, narrow banded filters. It is only necessary to substitute the apparant extinction coefficients of the substances with the proper filter for the true spectrophotometric extinction coefficients.

(3) Method of Hufner.[63] Hufner's method is very similar to that of Vierordt. The same simultaneous equations are used, but the final formula gives the *percentage composition* of the mixture in terms of the ratio (R) of the extinction coefficients of the mixture at two wave lengths.

Let $R = E_{am} / E_{bm}$

and per cent $X = 100\ X / X + Y$ (percentage concentration of X in the total colored solutes).*

From 7 and 8 one obtains the following equation by division and rearrangement:

$$\text{Per cent } X = \frac{100\ (E_{ay} - R\ E_{by})}{R\ (E_{bx} - E_{by}) + E_{ay} - E_{ax}} \qquad (11)$$

Several methods have been proposed for the determination of methemoglobin in the presence of oxyhemoglobin through the use of these formulas. Most of these depend upon the Hufner ratio of the extinction coefficients at 540 / 560,[64] which have been determined for both oxyhemoglobin and alkaline methemoglobin by Hari[39, 65] and Heilmeyer.[34] However, these ratios are so close together that a large change in the composition of a mixture is reflected by only a small change in the ratio, e.g., 0.43 for the complete conversion of oxyhemoglobin to methemoglobin. Both Heilmeyer and Drabkin and Austin[66] point out that the ratios at 576-575/560 mμ change approximately twice as much, for a given change in composition, as those at 540/560 mμ, and Heilmeyer[34] shows that the ratios at 576-575/590 have a far greater dispersion. The choice of wave lengths at 540 mμ and 560 mμ is doubly disadvantageous since Austin and Drabkin have demonstrated that this ratio is markedly influenced by pH variations.[67] Their data show that both the ratios at 576/560 and at 576/590 are much less disturbed by changes in the pH of the solution. The disadvantage of the 590 mμ measurement is that the light transmissions of both oxyhemoglobin and methemoglobin are changing very rapidly with wave length.

Because these methods depend to a variable extent upon the pH of the solution, Michel[68] has advised that the methemoglobin be converted to cyanmethemoglobin. The oxyhemoglobin is not changed by this procedure and the absorption spectra of both oxyhemoglobin[69] and cyanmethemoglobin are nearly independent of pH variations. The ratios of the extinction coefficients for these substances at 575 mμ and 560 mμ vary even more, for the same change in composition, than the corresponding ratios for oxyhemoglobin and methemoglobin. This method will be described in detail later.

For the determination of the total pigment in the mixture, the equations of Vierordt may be used. Drabkin and Austin[66] believe that greater accuracy is obtained if the total pigment is measured separately by the absorption at any wave length after conversion to cyanmethemoglobin by the addition of ferricyanide and cyanide.

The above methods are rapid but their accuracy is disturbed by any turbidity of the solution or the presence of a third pigment. To clarify the

*Hereafter percentage concentration refers to this concept. Concentration will refer to grams of the component in 100 c.c. solution.

solutions for the determination of the percentage methemoglobin, we fractionally precipitate with ammonium sulfate and use the filtrate. This procedure does not affect the methemoglobin-hemoglobin ratio of the mixture or cause any detectable loss in pigments.

Methods for the determination of sulfhemoglobin in oxyhemoglobin solutions have been worked out along similar lines. Prior to 1935, spectrophotometric methods could not be used since the absorption curve of sulfhemoglobin had not been determined. This curve has now been determined indirectly.[9] Our method for the determination of sulfhemoglobin depends upon the ratio of the extinction coefficients of the mixture of oxyhemoglobin

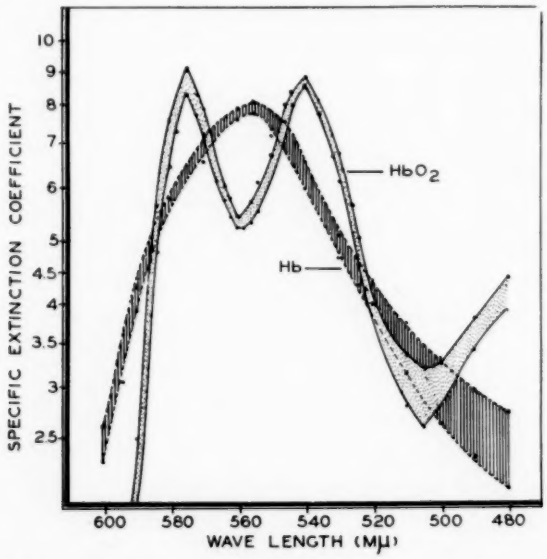

Fig. 1.—The "typical absorption spectra" of oxyhemoglobin and hemoglobin. Oxyhemoglobin curve plotted from the data of Hari,[39] Charnass,[41] Kennedy,[36] and Drabkin and Austin.[9, 67] Hemoglobin curve plotted from the data of Warburg, Negelein, and Christian,[39] Hari,[42] and Drabkin and Austin.[9] The shaded areas in each curve represent the maximum deviations found. *Ordinate:* Specific extinction coefficient for a 1 gm. per 100 c.c. solution. *Abscissa:* Wave length in mμ.

and sulfhemoglobin at 540 mμ / 620 mμ. The values of these extinction coefficients were taken from the data of Drabkin and Austin.[9] The absorption spectra of both compounds are only slightly influenced by pH changes but, as in the other methods, the solutions must be optically clear. Although the method requires the reading of the extinction at 620 mμ in concentrated solution and then the dilution of the solution in order to obtain the extinction at 540 mμ, it has the advantage of giving large changes in the ratio for very small changes in the percentage concentration of sulfhemoglobin.

(4) Method of Haurowitz.[25]—The *concentration* of each of two colored substances in solution can be calculated if the total concentration of pigment is known and the total extinction of the mixture is determined at any wave length.

Let C_t be the total concentration of pigment.

E_m, X, Y, etc., be as in equations 7 and 8.

From 5 and 6,

$$E_m = E_x X + E_y (C_t - X) \qquad (12)$$

and

$$X = \frac{E_m - E_y\ C_t}{E_x - E_y} \qquad (13)$$

Haurowitz[25] noted that the absorption of methemoglobin at 623 mμ was independent of the pH of the medium. He, therefore, proposed that the extinction coefficients at 623 mμ of a mixture of methemoglobin and oxyhemoglobin be determined before and after the conversion of all pigment to methemoglobin by means of ferricyanide. The total pigment is determined by the division of the extinction coefficient after ferricyanide addition by the specific extinction coefficient of pure methemoglobin. The concentration of methemoglobin in the original mixture is then obtained from formula 13, using the extinction coefficient of the mixture before ferricyanide as the E_m value. The method, however, is inaccurate because of the effect of turbidity and because the isobestic point may not be located exactly at 623 mμ.

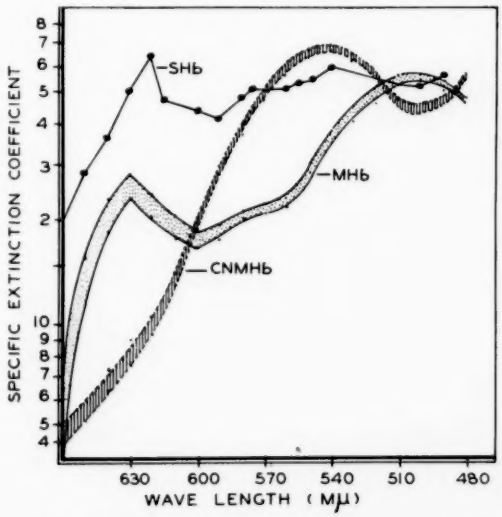

Fig. 2.—The "typical absorption spectra" of acid methemoglobin, cyanmethemoglobin, and sulfhemoglobin. Acid methemoglobin curve constructed from the data of Hari,[65] Haurowitz,[25] and Austin and Drabkin.[67] Cyanmethemoglobin curve from data of Haurowitz,[25] and Drabkin and Austin.[9] Sulfhemoglobin curve after Drabkin and Austin.[9] Ordinate and abscissa as in Fig. 1.

Austin and Drabkin[67] have advocated the use of a similar method for the determination of methemoglobin in oxyhemoglobin solutions. Their method requires the following measurements: The extinction coefficients of the solution at 630 mμ, 575 mμ, 560 mμ, and 540 mμ; the pH; and the concentration of total pigment by conversion to cyanmethemoglobin as previously described. The solutions must be optically clear and the absorption must be due only to methemoglobin and oxyhemoglobin. Turbidity or a third pigment will falsify the result.

(5) Authors' method. If the composition of one colored component in a mixture can be altered without changing any of the other components, the concentration of that substance may be determined by measuring the extinction coefficient of the mixture before and after the alteration.

Let

E_{ax} be the specific extinction coefficient of component X at wave length a.

E_{ax}' be the specific extinction coefficient of substance X′ which is formed from X by the addition of Z.

E_m be the observed extinction coefficient of the original mixture.

E_z be the observed extinction coefficient of the mixture after the addition of Z.

Then

$$\text{Conc. X} = \frac{E_m - E_z}{E_{ax} - E_{ax}'} \qquad (14)$$

This method has been applied to the photoelectric photometer determination of methemoglobin in oxyhemoglobin solutions by Evelyn and Malloy[70] and independently by us to the spectrophotometric determination. Both are based upon the fact that the addition of cyanide quantitatively converts methemoglobin to cyanmethemoglobin without affecting the absorption spectra of either sulfhemoglobin or oxyhemoglobin. Because the spectrum of methemoglobin varies with the pH and ionic strength, it is necessary to buffer the solution to a known pH and to a reasonably constant salt concentration. The buffer is also necessary to prevent the cyanide from changing the clarity of the solution. With these restrictions, the methods are simple; they allow the use of uncleared solution and may be used in the presence of other pigments which are unaffected by cyanide.

METHODS

A. The Determination of Methemoglobin in Oxyhemoglobin Solutions by the Use of the Hufner Ratio for Cyanmethemoglobin and Oxyhemoglobin

One volume of solution (whole oxalated blood) is hemolyzed by the addition of a few milligrams of saponin. Two volumes of half saturated ammonium sulfate are added. The mixture is cooled and filtered. The filtrate is diluted with distilled water until the total pigment concentration is approximately 0.1 gm. per cent. The final dilution of whole oxalated blood should be 1:100. Approximately 2 to 3 mg. of dry potassium cyanide are added, and the extinction coefficients are read at wave lengths 575 and 560 mμ. The ratio (R) of the extinction coefficient at 575 mμ to that at 560 mμ is calculated. The fraction of total hemoglobin in the form of methemoglobin is given by the following equation.

$$\text{MHB/Total HB} = \frac{9.06 - 5.39\ R}{0.53 + 4.66}$$

This value may be obtained directly from the calibration curve in Fig. 3.

It has been stated previously that the R value of a solution is independent of concentration. If one is merely interested in the fraction of total hemoglobin in the form of methemoglobin, the dilutions may be carried out very roughly. The method is, therefore, useful for the rapid determination of the percentage composition of a series of hemoglobin solutions. To determine the total hemoglobin, the dilutions must be made accurately. The total concentration may be calculated from the following equation:

$$\text{Grams per cent total pigment} = \frac{4.66\ E_{560} + 0.53\ E_{575}}{29.91} \times \frac{1}{\text{Final dilution}}$$

The results may be checked by converting all the pigment to cyanmethemoglobin and then determining the extinction coefficient at 540 mμ. After the readings at 575 and 560 have been taken, approximately 3 mg. of dry potassium ferricyanide are added to the material in the spectrophotometer cup. A few minutes later, an additional 2 to 3 mg. of potassium cyanide are added. The extinction coefficient is read at 540 mμ.

$$\text{Grams per cent total pigment} = \frac{E_{540}}{6.9} \times \frac{1}{\text{Final dilution}}$$

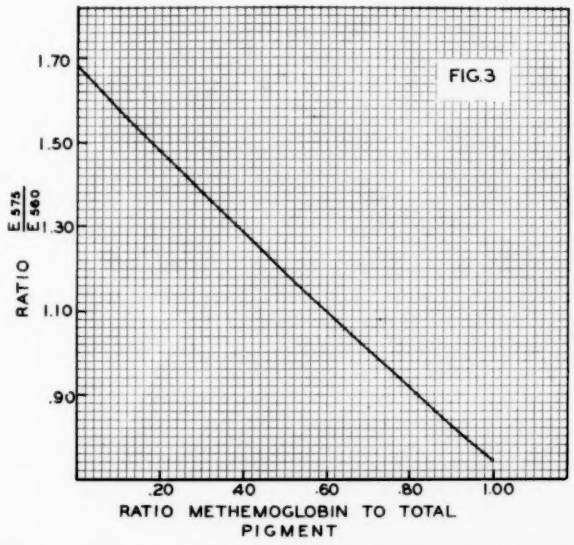

Fig. 3.—The values of the ratio of the extinction coefficients at 575 mμ to that at 560 mμ for varying mixtures of cyanmethemoglobin and oxyhemoglobin.

B. The Determination of Methemoglobin and Oxyhemoglobin by Means of the Change in Extinction on Conversion to Cyanmethemoglobin

One cubic centimeter of whole oxalated blood is hemolyzed by the addition of a few milligrams of saponin. It is then diluted to approximately 23 c.c. with distilled water. One and one-fourth cubic centimeters of 1.0 molar phosphate buffer* are added, and the solution diluted to the final volume of 25 c.c. Without filtering, the extinction coefficient is determined at 634 mμ. Two to 4 mg. dry potassium cyanide are added to the solution in the spectrophotometer cup and, one minute later, the extinction coefficient at 634 mμ is again determined. It is to be noted that it is unnecessary to read the solution through both beams of the photometer, since one is only interested in the difference between the two extinctions and not their absolute values. The cells and plungers of the spectrophotometer must be carefully washed after the use of cyanide, since the next sample may be partly changed by the

*1 M 7.4 Phosphate buffer: 114.8 gm. anhydrous Na_2HPO_4 and 26.15 gm. anhydrous KH_2PO_4 per liter.

1 M 6.2 Phosphate buffer: 26.42 gm. Na_2HPO_4 and 110.9 gm. KH_2PO_4 per liter.

The factors for two phosphate buffers have been given because it is sometimes necessary, as in tissue respiration experiments, to buffer the solution to pH 7.4. At this pH, approximately 1/3 of the methemoglobin is in the alkaline form and the factor is, therefore, smaller than that at pH 6.2.

cyanide before the first extinction is read. The concentration of methemoglobin is given by the following formula:

$$\text{Grams per cent methemoglobin} = \frac{E_{634} - E_{634} \text{ with cyanide}}{F}$$

where F is 0.0728 when pH of solution is 7.4, or 0.0860 at pH 6.2.

To determine the total hemoglobin and methemoglobin concentration, approximately 5 mg. of dry potassium ferricyanide are added to another portion of the diluted blood. After mixing, the solution is allowed to stand five minutes. The extinction coefficients at 634 mμ are then obtained before and after the addition of cyanide. The total pigment is calculated by the same formula as above.

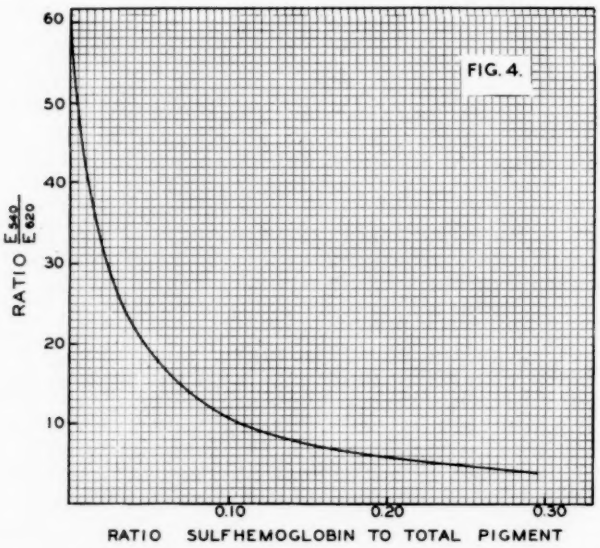

Fig. 4.—The values of the ratio of the extinction coefficients at 540 mμ to that at 620 mμ for varying mixtures of sulfhemoglobin and oxyhemoglobin.

C. The Determination of Sulfhemoglobin

Methemoglobin is first determined by a modification of the preceding method. The blood is, therefore, clarified by the use of 2 M pH 7.4 phosphate buffer* instead of ammonium sulfate. The greater ionic strength of this solution necessitates a change in the factor used in the calculation of methemoglobin concentration. After the extinctions at 634 mμ have been determined, the ratio of the extinction coefficients at 540 mμ to that at 620 mμ is obtained in order to calculate the sulfhemoglobin concentration according to the usual formulas. The presence of the cyanide does not affect the extinctions of either the oxyhemoglobin or sulfhemoglobin, but corrections must be introduced to account for the presence of the cyanmethemoglobin. If methemoglobin is not present in the original solution, the first part of the procedure and the corrections may be omitted.

One volume of solution (whole oxalated blood) is hemolyzed by the addition of a few milligrams of saponin. Two volumes of 2 molar pH 7.4

*2 M pH 7.4 Phosphate buffer: 52.30 gm. KH_2PO_4 and 281.68 gm. K_2HPO_4 per liter.

phosphate buffer are added, and the mixture is filtered. Two cubic centimeters of the filtrate are diluted to 10 c.c. with distilled water. The extinction is determined at 634 mμ before and after the addition of 2 to 4 mg. of potassium cyanide. The extinction coefficient at 620 mμ is then determined. One cubic centimeter of the material in the spectrophotometer cup is now diluted to 10 c.c. (making a 1:150 dilution of the original blood), and the extinction coefficient is determined at 540.

Calculations:

$$\text{Grams per cent methemoglobin} = \frac{E_{634} - E_{634} \text{ with KCN}}{0.126}$$

$$\text{Corrected 620 extinction} = 15\ E_{620} - 0.09 \times \text{gm. per cent MHB}$$
$$\text{Corrected 540 extinction} = 150\ E_{540} - 6.9 \times \text{gm. per cent MHB}$$

From the corrected readings, the ratio (R) of the extinction at 540 to that at 620 is calculated and used in the following formula to determine the ratio of pigment (exclusive of methemoglobin) in the form of sulfhemoglobin.

$$\frac{\text{Sulfhemoglobin}}{\text{Total hemoglobin}} = \frac{8.76 - 0.144\ R}{6.26\ R + 2.86}$$

The percentage composition (exclusive of methemoglobin) may be read directly from the calibration curve in Fig. 4.

The total pigment concentration in grams per 100 c.c. of the original solution is calculated from the corrected extinction coefficients by means of the following equation:

$$\text{Grams per cent total pigment} = \text{Grams per cent methemoglobin} + \frac{6.26\ E_{540} + 2.86\ E_{620}}{55.2}$$

EXPERIMENTAL

The total hemoglobin content of freshly drawn oxalated human blood was determined after reduction with sodium hydrosulfite by the carbon monoxide capacity method of Van Slyke and Hiller.[47] Solutions of these bloods were then prepared in accordance with the methods of determination previously described. The extinctions at the wave lengths used in the various methods were then obtained on the Bausch and Lomb Universal Spectrophotometer.* Another sample of the original blood was then checked for the active hemoglobin content by the carbon monoxide method without preliminary reduction with hydrosulfite. In this manner, the absence of methemoglobin in the original sample was assured. Spectroscopic analyses ruled out the presence of other abnormal pigments. One experiment (Blood No. 9, Table I) was performed on purified hemoglobin prepared by shaking washed red blood cells with toluene, centrifuging off the cell debris, and dialyzing the solution against distilled water for several days. The results are tabulated in Table I, and the values found by other investigators on differently prepared solutions are given for comparison.

*The technique of using this apparatus is fully described by Heilmeyer[34] and Kennedy.[35] For the work herein reported, the apparatus was placed in an air-conditioned darkroom at 20° C. The spectroscope was calibrated by means of the sodium light and the arc spectra of lithium, potassium, and magnesium. The solutions were observed in both beams of light, and the arithmetical means of the observed extinctions were calculated. The depth of solution was such that the observed extinctions were between 0.50 and 1.50, which Kennedy found to be the optimum range. The spectroscope objective slit was reduced to a minimum, and the portion of spectrum observed was always less than 4 mμ.

TABLE I

EXTINCTION COEFFICIENTS OF OXYHEMOGLOBIN AND CYANMETHEMOGLOBIN

	BLOOD SAMPLES										VALUES IN LITERATURE					
	1	2	3	4	5	6	7	8	9	AVERAGE	HARI[39]	NEWCOMER[71]	DRABKIN AND AUSTIN[9]	KENNEDY[35]	CHARNASS[41]	MARTINI[72]
CO combining power	22.36	19.36	21.37	19.57	24.13	22.35	17.74	18.95	24.85	--	--	--	--	--	--	--
Hemoglobin (gm. %)	16.66	14.43	15.93	14.60	17.99	16.66	13.23	14.12	18.52	--	--	--	--	--	--	--
HbO_2 E_{540} 1:100 dil.	1.440	1.255	1.374	1.302	1.595	1.464	--	--	--	--	--	--	--	--	--	--
HbO_2 E_{540}	8.65	8.70	8.63	8.92	8.87	8.79	--	--	--	8.76	8.50	8.79	8.80	8.59	8.55	9.14
HbO_2 E_{560} 1:100 dil.	0.896	0.766	0.842	0.801	0.978	0.903	--	--	--	--	--	--	--	--	--	--
HbO_2 E_{560}	5.38	5.31	5.28	5.49	5.44	5.42	--	--	--	5.39	5.28	5.34	5.28	5.26	5.18	5.55
HbO_2 E_{575} 1:100 dil.	1.498	1.296	1.412	1.352	1.653	1.508	--	--	--	--	--	--	--	--	--	--
HbO_2 E_{575}	9.00	8.98	8.87	9.26	9.19	9.06	--	--	--	9.06	8.35	9.36	9.06	8.70	8.64	9.43
HbO_2 E_{620} 1:10 dil.	0.243	0.209	0.228	0.212	0.250	0.245	--	--	--	--	--	--	--	--	--	--
HbO_2 E_{620}	0.146	0.145	0.143	0.145	0.139	0.147	--	--	--	0.144	0.116	0.467	0.33	--	--	--
CNMHb E_{575} 1:100 dil.	--	0.621	0.692	0.644	0.804	0.750	0.581	--	--	--	--	--	--	Haurowitz[25]	--	--
CNMHb E_{575}	--	4.30	4.34	4.41	4.47	4.50	4.39	--	--	4.40	--	--	4.46	4.17	--	--
CNMHb E_{560} 1:100 dil.	--	0.833	0.928	0.878	1.088	0.992	0.784	--	--	--	--	--	--	--	--	--
CNMHb E_{560}	--	5.77	5.82	6.01	6.05	5.95	5.92	--	--	5.92	--	--	5.97	5.63	--	--
F pH 7.4 0.05 M buffer	1.801	--	1.807	1.831	1.821	--	1.845	1.812	1.809	1.82	--	--	--	--	--	--
F pH 6.2 0.05 M buffer	2.138	--	2.165	2.150	2.170	--	2.110	2.152	2.160	2.15	--	--	--	--	--	--
F pH 7.4 0.27 M buffer	--	--	--	--	--	--	--	--	--	1.89*	--	--	--	--	--	--

Note: F = Change in *E* at 634 mμ on converting methemoglobin to cyanmethemoglobin. *E* given for 1 gm. in 100 c.c. of solution.

*Value obtained by determining F of 5 solutions at pH 7.4 at 0.05 M and 0.27 M buffer.

Tables II and III show the results obtained with known solutions of methemoglobin and oxyhemoglobin. They were prepared by mixing definite amounts of two solutions whose oxyhemoglobin and methemoglobin contents were determined by the gasometric carbon monoxide method of Van Slyke and Hiller.[47] Table III gives the results obtained by means of the difference in extinction before and after conversion of the methemoglobin to cyanmethemoglobin, while Table II gives the results obtained by means of "Hufner ratio" for oxyhemoglobin and cyanmethemoglobin. The former method is considerably more accurate than the latter and has the advantage that the accuracy can be improved by reading the extinctions in more concentrated solutions when the amount of methemoglobin is small. Both methods require few reagents, and many analyses can be carried out within an hour.

TABLE II

DETERMINATION OF METHEMOGLOBIN BY MEANS OF THE RATIO OF EXTINCTION COEFFICIENTS AT 575 AND 560 mμ AFTER ADDITION OF CYANIDE

SOLUTION	GASOMETRIC CARBON MONOXIDE METHOD			SPECTROPHOTOMETRIC METHOD	
	TOTAL HEMOGLOBIN (GM. %)	METHEMOGLOBIN (GM. %)	PER CENT TOTAL PIGMENT PRESENT AS METHEMOGLOBIN	RATIO EXTINCTION AT 575 TO 560 mμ	PER CENT TOTAL PIGMENT PRESENT AS METHEMOGLOBIN
1	13.2	0.3	2.3	1.66	2.0
2	18.5	5.9	31.9	1.35	33.0
3	13.9	1.0	7.2	1.59	8.6
4	14.6	1.7	11.6	1.56	12.0
5	15.2	2.4	15.8	1.53	14.8
6	15.9	3.1	19.5	1.48	19.8
7	16.5	3.8	23.0	1.44	23.8
8	17.2	4.5	26.2	1.41	26.9
9	17.9	5.2	29.0	1.37	31.0

TABLE III

DETERMINATION OF METHEMOGLOBIN BY CHANGE IN EXTINCTION UPON CONVERSION TO CYANMETHEMOGLOBIN

SOLUTION	GASOMETRIC CARBON MONOXIDE METHOD			SPECTROPHOTOMETRIC METHOD		
	TOTAL HEMOGLOBIN (GM. %)	METHEMOGLOBIN (GM. %)	PER CENT TOTAL PIGMENT PRESENT AS METHEMOGLOBIN	TOTAL HEMOGLOBIN (GM. %)	METHEMOGLOBIN (GM. %)	PER CENT TOTAL PIGMENT PRESENT AS METHEMOGLOBIN
1	13.2	0	0	13.2	0	0
2	18.5	5.71	30.8	18.6	5.55	29.8
3	13.9	0.71	5.1	13.7	0.71	5.2
4	14.6	1.43	9.7	14.5	1.41	9.7
5	15.2	2.14	14.1	15.3	2.16	14.1
6	15.9	2.86	18.0	15.8	2.80	17.7
7	16.5	3.57	21.6	16.5	3.63	22.0
8	17.2	4.28	24.9	17.1	4.29	25.0
9	17.9	5.00	27.9	17.8	4.97	27.9

The procedure for sulfhemoglobin has not been checked because of the lack of methods. It would be pointless to check our method by means of other spectrophotometric methods which depend upon the same constants of Drabkin and Austin. Gasometric and other analytic methods are valueless for this purpose.[9]

SUMMARY

The interrelationships, properties, and reactions of the clinically important blood pigments have been reviewed. The methods for the determination of sulfhemoglobin and methemoglobin have been presented and critically discussed. Simple and accurate spectrophotometric methods for the determination of sulfhemoglobin, methemoglobin, and oxyhemoglobin in mixtures have been described.

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RELATION OF THE DIETARY CALCIUM-PHOSPHORUS RATIO TO IRON ASSIMILATION*

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CONSIDERABLE attention has been given to the possible relationship between infantile rickets and anemia. Findlay[1] was the first to show that anemia in rickets was due to complicating factors such as poor nutrition and not due directly to injury of the bone marrow. He showed that children fed good diets low in vitamin D developed rickets without showing anemia. Later McDonough and Borgen[2] found that the administration of vitamin D to infants suffering from anemia and rickets did not raise the hemoglobin level. The administration of iron salts to the rachitic infants without supplementing the diet with cod-liver oil raised the hemoglobin content of the blood to a satisfactory level.

Working with animals, Happ[3] found that a severe rachitogenic diet had no effect upon the hemoglobin level of either young or mature rats. On the other hand, diets low in calcium and vitamin D, but high in phosphorus, produced both rickets and anemia, a syndrome apparently comparable to that of human infantile rickets. The observation suggested that the hemoglobin regeneration was retarded by either high level of phosphorus or low level of calcium.

There is ample evidence[4, 5] that a high intake of iron will bind the phosphorus and make it unavailable to the animal. From a strictly chemical point of view there is no reason to suspect that high levels of phosphorus should prevent iron assimilation, yet Day and Stein[6] have produced a hypochromic anemia and polycythemia in rats on low calcium-high phosphorus rations.

That excess calcium will inhibit the assimilation of iron has been recognized by Kletzein[7, 8] who found that in addition to calcium carbonate the carbonates of sodium, potassium, beryllium, strontium, and barium exhibit this effect.

We have been studying the effect of the calcium and phosphorus ratio on iron assimilation and utilization for some time, and in this paper we wish to report some of this work, together with an attempt to correlate our results with those obtained by other workers.

EXPERIMENTAL

Rats were made anemic by the method of Elvehjem and Kemmerer[9]. When the animals were distinctly anemic, they were transferred to a dry ration

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containing varying amounts of calcium, phosphorus, iron, and copper. The basal ration had the following composition.

Corn, yellow	50
Corn, white	20
Casein	18
Lard	5
NaCl	1
Liver extract (Lilly)	2
$CaCO_3$	0-4
NaH_2PO_4	0-1
Thiamine	100 micrograms

Percomorph oil was administered orally at the rate of 2 drops per week.

Care was taken in the selection and grinding of corn to reduce the iron contamination to a minimum. Several attempts were made to purify the casein, but the method effective for the removal of the iron reduced the nutritive value of the casein. In the work reported, therefore, crude casein was used. The liver extract added some iron, but its use as a source of the vitamin B complex was essential.

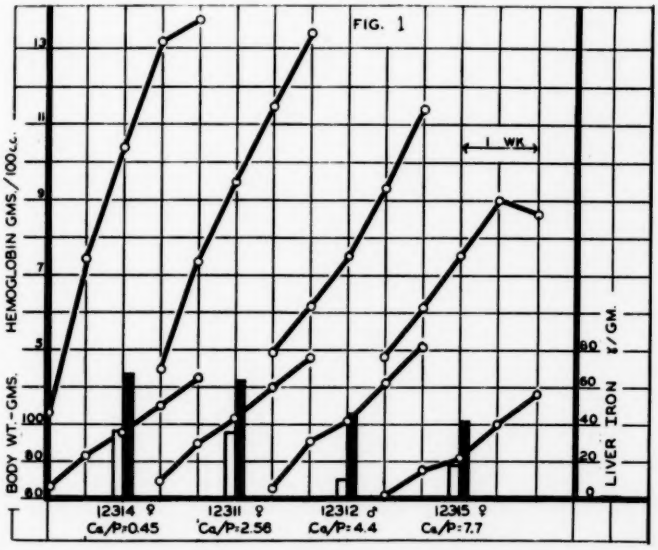

Fig. 1.—Upper curves represent hemoglobin response; lower curves, growth. Bars represent the level of total and available iron in the livers. Levels of minerals in the ration were: Fe = 139 μg per gm. and Cu = 9.5 μg per gm.

The basal ration never contained more than 39 micrograms of available iron per gram. Additional iron to make the desired intake was added as ferric chloride or ferric pyrophosphate. The copper content was varied by the addition of copper sulfate. The calcium and phosphorus were added as calcium carbonate, and sodium diphosphate, respectively.

The animals were housed in draw cages with mesh bottoms of two squares to the inch. Feed and water were given in earthen dishes with aluminum guard covers to reduce waste. The animals were weighed, and the hemoglobin content of the blood determined biweekly. They were continued on experiment for two weeks, at which time they were killed by decapitation and their livers

removed for analysis of total iron by the method of Lintzel[10], and available iron by the method of Borgen and Elvehjem[11].

At the culmination of the experiment the pH was determined on three segments of the gastrointestinal tract of each animal. These segments were (a) the duodenum and one-half the jejunum, (b) one-half the jejunum and the ileum, (c) the colon and the cecum. The segments were removed, their contents washed into a clean tube, diluted to 15 c.c., and the pH read on a Coleman pH electrometer.

The effect of the calcium-phosphorus ratio on iron utilization when the iron and copper levels remained constant is shown in Fig. 1. The only variable in these experiments was the calcium and phosphorus levels. Hemoglobin regeneration and iron storage in the liver were greatest on the lowest calcium-phosphorus ratio (0.45). As the calcium-phosphorus ratio was increased, there was a corresponding decrease in both hemoglobin regeneration and iron storage in the liver. Since these data are representative of the results in general, more extensive data are omitted. It serves to show that within the range of this study, phosphates have a stimulating and not an inhibitory effect upon iron assimilation.

The rapid hemoglobin response obtained on the dry ration made it seem desirable to compare the hemoglobin response on this ration with that on mineralized milk. Results shown in Fig. 2 indicate that the hemoglobin response on mineralized milk was greater than that on the dry ration which had higher calcium-phosphorus ratios, but less than that on the ration with the calcium-phosphorus ratio of 0.45. Growth on the mineralized milk used was definitely inferior to that on the dry rations.

Since blood volume correlates roughly with the body weight in a growing animal, it seemed possible that the rats on mineralized milk might be producing less hemoglobin than a litter mate which grew more rapidly, and yet show a higher hemoglobin level. Since such a condition was not easily shown by hemoglobin changes alone the following procedure was employed.

The two places for storage of iron, which have been shown to be greatly affected during the production and cure of anemia, are the liver and the hemoglobin of the blood. From the standpoint of quantity, the hemoglobin accounts for the greater portion of the iron, but the liver iron serves to indicate whether iron has not been assimilated or merely not converted to hemoglobin.

The amount of available iron consumed by the animal was determined by analyzing the ration and multiplying by the amount of ration consumed. The amount utilized was determined as follows:

To determine the amount of iron used in the formation of hemoglobin, the levels of hemoglobin, as determined by analysis, were multiplied by the body weight, the blood volume, and the percentage of iron in the hemoglobin molecule. An average value of 8.1 per cent was taken as the blood volume of the rat, and 0.335 per cent as the amount of iron in hemoglobin. Then

$$0.081 \times \text{Body weight} \times \text{Hemoglobin level} \times 0.335 \text{ per cent} = \text{Hemoglobin iron.}$$

Subtracting the initial value from that of the final value would then give an expression for the change of iron due to the change of hemoglobin:

$$\text{Final } Fe_{Hb} - \text{Initial } Fe_{Hb} = \triangle\ Fe_{Hb}$$

To account for the change in liver iron it was necessary to know approximately the level of iron in the livers of anemic rats, and what percentage of the body weight was represented by the liver. This was accomplished by analyzing livers from 2 rats taken from litters at the time the remaining litter mates were started on therapy. Results were consistent enough to make such a method seem justified. For this reason, average values for total iron and per cent of body weight represented by the liver were employed. The final values, as determined by analysis, less the initial calculated values, expressed the relative change in liver iron.

Final liver weight × Level of liver iron − Initial body weight × 5.83% × 17.5 μg/gm. = Change in liver iron.

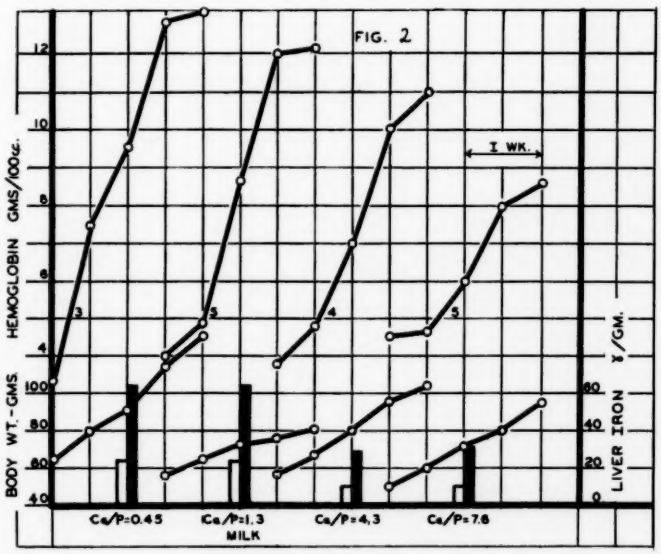

Fig. 2.—Upper curves represent hemoglobin response; lower curves, growth. Bars represent the level of total and available iron in the livers. Iron was fed at a level of 89 μg per gm. and copper at a level of 9.5 μg per gm. Arabic numerals adjacent to the hemoglobin curves indicate the number of animals in the group.

The change in iron due to hemoglobin formation plus the change in iron due to liver storage, divided by the available iron eaten and multiplied by 100 represents the percentage utilized.

$$\frac{\triangle\ Fe_{liver} + \triangle\ Fe_{hemoglobin} \times 100}{Fe_{(available)} \times \text{gm. diet eaten}} = \text{Per cent utilized.}$$

Table I summarizes the results obtained when this procedure was applied to the data shown in Figs. 1 and 2 as well as the data from a number of other animals. The results indicate that the utilization of the iron on the diet containing Ca/P=1.87 to Ca/P=2.56 was approximately equal to that on milk. When, however, the calcium-phosphorus ratio was lowered to 0.45,

TABLE I

RAT NO.	SEX	Ca	P	Ca/P	Cu	AVAILABLE Fe	DIET EATEN	Fe EATEN	Δ Fe Δ Hb	Δ Fe$_L$	COLON pH	UTILIZED
		%	%		μg/gm.	μg/gm.	gm.	mg.	mg.	mg.		%
12301	F	0.29	0.648	0.45	9.0	138	130	17.9	3.45	0.301	5.75	20.9
12314	F	0.29	0.648	0.45	9.0	138	152	21.0	4.52	0.226	5.25	22.6
12322	M	0.29	0.648	0.45	9.0	138	167	23.1	4.63	0.215	5.95	21.0
11941	F	0.40	0.649	0.62	9.0	138	122	16.9	3.38	0.369	5.86	22.2
11961	M	0.40	0.649	0.62	9.0	138	93	12.9	2.77	0.212	5.62	23.1
11971	F	0.40	0.649	0.62	9.0	138	125	17.3	3.65	0.489	6.10	23.8
11942	F	1.20	0.64	1.87	9.0	138	108	14.9	2.82	0.121	6.80	19.7
11972	M	1.20	0.64	1.87	9.0	138	145	20.0	3.70	0.378	6.97	20.4
12311	F	1.64	0.64	2.56	9.0	138	178	24.6	4.18	0.228	6.90	17.9
12324	F	1.64	0.64	2.56	9.0	138	156	21.6	3.72	0.101	6.90	17.7
12312	M	1.78	0.404	4.40	9.0	138	147	20.3	3.62	0.136	6.9	18.4
12321	M	1.78	0.404	4.40	9.0	138	166	22.9	3.62	0.150	6.2	16.4
11963	M	1.62	0.404	4.10	9.0	138	92	12.8	2.02	0.107	6.5	14.8
12303	M	3.07	0.402	7.65	9.0	138	147	20.3	2.65	0.124	6.55	13.5
12315	F	3.07	0.402	7.65	9.0	138	138	19.0	1.95	0.066	7.18	10.6
12333	F	3.07	0.402	7.65	9.0	138	125	17.3	1.78	0.076	6.90	10.7
11683	M	Milk		1.3	≋	≋		18.6	3.16	0.246		18.4
11674	F	Milk		1.3	≋	≋		18.0	3.12	0.067		17.7
11691	F	Milk		1.3	≋	≋		12.5	2.14	0.045		17.4

the utilization of iron was markedly increased. There appears considerable latitude in the calcium-phosphorus ratio over which there is little variation in the utilization, especially in the range normally employed in rations (Ca/P=1.3 to 4.5).

The effect which the calcium-phosphorus ratio had on iron utilization is shown graphically in Fig. 3. This evidence, in addition to that presented in Figs. 1 and 2 and Table I, establishes the fact that iron utilization decreases with the increase of the calcium-phosphorus ratio and that maximum iron utilization occurs when the calcium-phosphorus ratio is less than that of whole milk.

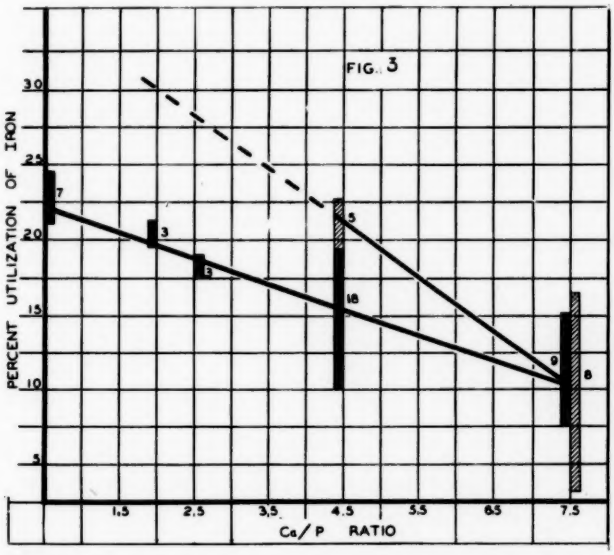

Fig. 3.—Iron level = 139 μg per gm. Solid bars indicate the range of data where Cu = 9 μg per gm., lined bars where Cu = 50 μg per gm. (in the ration). Adjacent numerals indicate the number of animals from which the data were obtained.

The effect which the calcium-phosphorus ratio has on iron utilization in the presence of additional copper is also shown in Fig. 3. The results indicate that the higher calcium-phosphorus ratios might also be retarding the utilization of copper. The data are insufficient to warrant any conclusions at this time, but work is being continued in this laboratory to check this observation.

The fact that calcium salts in excess make iron salts less available to an animal while phosphates have the opposite effect, indicated the possibility that there might be a difference in the pH of the gastrointestinal tracts of the animals. Results indicated little change in any segment of the tract except that of the colon-cecum segment. As is shown in Table I there was a marked lowering of the pH of the segment on the low calcium-phosphorus ratio. When calcium was added to the ration, the pH was again established between 6.5 and 7.2 which is in agreement with the observations of Robertson[12] who assigns to calcium the role of maintaining the pH of the colon at approximately 7.0.

DISCUSSION

The data here presented clearly demonstrate that high levels of calcium carbonate retard iron utilization and that monobasic sodium phosphate has

the opposite effect. That this was utilization and not merely hemoglobin formation was demonstrated by the fact that both hemoglobin regeneration and iron storage in the liver increased as the calcium-phosphorus ratio decreased.

The danger of considering the hemoglobin level as the sole index of iron assimilation is evident when we consider the role copper plays in the regeneration of hemoglobin. Copper does not influence the assimilation of iron but functions in its conversion to hemoglobin. Using the hemoglobin level alone as an index of iron utilization would not be accurate if copper were lacking. Since the formation of hemoglobin is a relatively complex process, there may be many factors which will retard it, among them a high level of phosphorus.

Day and Stein[6] studied the relation of calcium and phosphorus to iron utilization on the low mineral ration of Orten, Smith, and Mendel. They state ". . . in rats on a low mineral ration containing a relative excess of phosphorus, the mild polycythemia and anemia which occur are attributable to the presence of relatively excess phosphorus, and that the effect of calcium in preventing the hematopoietic abnormality resides only in its ability to bind phosphorus, thus permitting the dietary iron to be used for hematopoiesis instead of being excreted presumably as a phosphate."

There seems little doubt that they produced abnormal effects with extreme disturbances of the mineral level. For the most part, they used greater extremes in the calcium-phosphorus ratios than we employed. Our data then are not directly comparable, but in light of the data which we obtained it seems possible that in their experiment the abnormality appeared not as a result of poor iron assimilation but rather as a result of some other effect upon hemoglobin maintenance.

Our results, together with those of other workers reviewed here, seem to indicate that within limits ordinarily reached in most diets, calcium impedes the absorption of iron, while phosphorus has a stimulating effect.

SUMMARY

1. A ration is described which, when supplemented with iron and copper, gives equally as rapid growth and hemoglobin regeneration in anemic rats as mineralized milk.

2. Studies on the calcium-phosphorus ratio indicated that a low calcium-phosphorus ratio was favorable to iron utilization since maximum utilization occurred at Ca/P = 0.45, with decreasing utilization as the Ca/P ratio increased to 7.65.

3. Studies on the pH of the intestinal tract showed that the pH of the colon of rats on a diet with Ca/P = 0.45 was distinctly lower (5.2 to 6.1) than those on diets with a higher Ca/P ratio in which the pH of the colon was 6.2 to 7.2.

4. The stimulating effect obtained with the higher levels of copper indicated that copper is not utilized as completely from such a dry ration as from milk.

5. A method of expressing the precentage of iron utilized by anemic rats during therapy is described.

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TRANSFUSIONS AND POLYCYTHEMIA IN NORMAL AND TUMOR-BEARING RATS

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ANY of the familiar types of transplantable tumors of rats and mice, situated most conveniently in a leg, may be subjected to asphyxia by temporary ligation of the leg, with two chief results: (a) dark hemorrhagic congestion of the tumor in contrast with the bright reactive hyperemia of the normal tissues after release of the ligature; (b) extensive necrosis of the tumor, sometimes to the point of actual cure, without permanent damage of the normal tissues.[1] As the cause of the tumor death evidently consists in the vascular or inflammatory reaction,[2] inquiry is naturally turned either to intensifying this result or producing it by some other means. One possible factor for consideration is the increased corpuscle content and viscosity of the blood following such ligations.[3] It is conceivable that the desired effects may be assisted if the corpuscle content be still further increased, and perhaps also if the reduced blood volume resulting from ligation be changed to an increased blood volume.

Such a change is opposite to the usual anemia of cancer. The latter, however, is not invariable, for normal blood counts are possible with early cancer and sometimes in the later stages.[4] On the other hand, clinical polycythemia offers some highly suggestive facts, since in the severe form it often leads to stasis and consequent ulceration or gangrene in the extremities or other places of sluggish circulation.[5] Assuming that the makeshift circula-

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tion of the tumor stroma constitutes a vulnerable point in the vascular system, it becomes probable that polycythemia will intensify the effects of ligation and may possibly set up a spontaneous process of similar character. Encouraging in this connection is the well-known fact that spontaneous recessions in tumors are often initiated by the occurrence of hemorrhages and infiltrations. These theoretical suggestions are not altogether supported by an inquiry into the possible incompatibility of cancer and polycythemia, because the actual combination is sometimes reported in clinical literature.[6] The rarity of this association, however, still permits the speculation that it exists only with special tumors or under special conditions, or that there may be some degree of true antagonism which may be utilized therapeutically. A rather long and extensive investigation will be reported here only in brief outline, presenting some new observations on both normal and tumor-bearing rats.

NORMAL CONTROLS

Rats, weighing between 100 and 200 Gm., will often tolerate transfusions of as much as 10 to 15 c.c. of heparinized blood, or 20 to 25 c.c. may sometimes be given in one day if divided into 2 or 3 injections. A large series of experimental findings may be summarized as follows:

1. Young rats, not fully grown, are sometimes exceptionally sensitive to transfusions and die either immediately or within a short time after receiving as little as 2 or 3 c.c. of blood. No investigation of blood groupings[7] was made. Some special peculiarity seems to exist in these animals, while, on the other hand, the high tolerance of adult rats generally holds good for blood from other strains, including colored rats.

2. Acute death from excessively large transfusions never occurs by heart failure, but always in one of two forms: (a) Apnea. This is adequately explained by the intense pulmonary congestion at autopsy in such cases. But it can also be proved that a needle inserted quickly in the heart or a jugular vein can withdraw more blood than was injected, so as to relieve all plethora and all visible pulmonary engorgement while the heart is still beating, and yet respiration or life is not restored. (b) Liver congestion. This is a remarkable phenomenon, occurring especially in rather young rats. It is evidently not due to any chemical factor but strictly to the quantity of blood. When a certain limit of injection is reached, the liver becomes rather suddenly swollen and tense, so that it is plainly palpable like a hard ball through the abdominal wall. This condition is confirmed at autopsy, when the congestion in the lungs and other viscera is found only moderate, and the spleen (except in one isolated instance) is found noticeably small and free from congestion. Here also the full amount of injected blood, or even more, can be withdrawn by quick aspiration of the heart or a jugular vein while the heart is still beating. The liver swelling and all plethora elsewhere can thus be completely relieved, but death is not prevented. A few trials of epinephrine were also unsuccessful. In either (a) or (b) there is no striking congestion in the brain or spinal cord.

3. After transfusion of too much blood, in either single or divided injections, death occurs after a number of hours or days from pulmonary congestion

or pneumonia. The symptoms are weakness and small quantities of blood-tinged fluid about the nares, evidently indicating pulmonary edema. Only a few hours after an excessive transfusion the lungs may be in a state resembling red hepatization. The slower deaths following smaller or divided transfusions are explained by varying degrees of pulmonary congestion, edema, and pneumonia.

4. Less numerous transfusions were given of separated corpuscles or plasma. Without precise quantitative comparisons, it can only be stated that the animals tolerate a somewhat smaller quantity of corpuscle sediment and a slightly larger quantity of plasma than of whole blood. The ultimate results are similar, except that with corpuscles the congested lungs and other viscera are found very red and with plasma very pale. A similar state of fatal pale pulmonary congestion is also produced by repeated infusions of artificial plasma made with gum acacia.

5. Transfusions of 5 to 10 c.c., repeated at one-, two-, or three-day intervals, are tolerated by different rats for about 4 to 7 times. Death then comes from the lungs as above described. In this way, the most resistant rats can be kept for two to three weeks in a state of plethora with red blood cell counts of 12 to 14 million, with eyes noticeably red and bulging, and with skin perceptibly pink and reddening excessively on slight irritation.

Ligations of Legs.—According to the purely mechanical theory of shock, plethoric rats should be able to endure longer ligations than normal animals. The opposite is true, however, since the plethoric animals are liable to die within four or even three hours of ligation of one hind leg, evidently because of subnormal general strength. Congestion in the ligated limb is obviously increased. When the tourniquet is placed low enough to avoid fatal shock, an increased tendency to gangrene seems demonstrable with sufficiently long ligations, but the time limits were not accurately determined.

TUMOR EXPERIMENTS

The following is a brief summary of results of over 100 experiments, comprising all three types of rat tumors used (Crocker sarcoma No. 39, Flexner-Jobling carcinoma, and Walker tumor), and single or repeated transfusions, generally of whole blood but of corpuscle sediment or plasma in a smaller number of cases.

Sufficiently large transfusions can of themselves produce extreme congestion in tumors, apparently more marked than in the lungs or any other organs. An important distinction is that this congestion is often followed by extensive necrosis in the tumors, but never in any normal organ. These effects are greatly increased by comparatively short ligations, of one-half to three hours. Such treatment readily produces complete sloughing of the tumor as far as can be judged by gross examination, while the normal leg tissues react with only moderate inflammation. Plasma, whole blood, and corpuscles form an ascending series for the production of all the above changes. Permanently curative results could not be judged because all animals treated sufficiently to cause apparently complete necrosis of tumors died within a few days from shock or pulmonary congestion. Because of the seemingly en-

couraging local results, the experiments were continued through a large series of transfusions as mentioned, also injections of gum arabic and of plain saline or glucose solutions, also with arsenic combinations and other expedients, without escaping the dilemma of death from pulmonary congestion when the transfusions were too large or from shock when the ligations were too long. While these experiments seemed to conform to the theory that a tumor constitutes a vulnerable point in the circulation, the essential difficulty was encountered in the almost equal vulnerability of the lungs.

AUTOGENOUS POLYCYTHEMIA

As the fatal consequences of the plethora produced by transfusions in rats are altogether different from the conditions in clinical polycythemia, further series of experiments were performed with the polycythemia produced by administration of metals (iron, copper, cobalt, manganese) as described by several authors.[8] In the successful cases, there was no difficulty in keeping rats for a number of weeks with counts of 12 to 16 million red blood cells without any of the pulmonary or other dangers which occur with transfusions. The principal observations can be summarized as follows:

1. The local and constitutional effects of ligation of legs appear to be somewhat augmented with the autogenous polycythemia as compared with the normal state. No numerical expression of the difference was attempted, but it is far less than with transfusions.

2. Animals with this metal polycythemia, and, on the other hand, animals made more or less anemic[9] by repeated bleedings, showed no significant changes in the number of "takes" after tumor inoculations.

3. As the result of numerous failures from various causes, only 19 rats were finally obtained with the desired combinations of marked polycythemia with a well-established tumor. Ligations for various periods produced distinctly greater hemorrhagic congestion than in normal animals, but this was less than in the transfused animals, and in particular the necrosis was much less. There was perhaps slightly less tolerance for ligation than in normal animals, and any increase in the actual breakdown of tumors was doubtful. No permanent cures were obtained in this comparatively small series.

In general, it must be concluded that polycythemia per se does not display any important degree of antagonism to tumor growth. The special congestion and subsequent necrosis of tumors in connection with large transfusions seem to be due to general circulatory failure. It seems doubtful if the desired tumor-killing effect could be obtained in any other species[10] which might stand the transfusions better than rats.

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DOES HEMOLYTIC STREPTOCOCCUS INFECTION OR SULFANILAMIDE AFFECT THE ALPHA HEMOLYSIN OF SERUM?*

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CERTAIN observations have been made in the course of routine tests which I feel should be called to the attention of the medical profession and particularly to the notice of those who specialize in serologic and immunologic studies.

In routine blood grouping practice, rapid hemolysis of incompatible cells without agglutination is not commonly encountered. Since Moss' clarifying work,[1] emphasis has been placed on the agglutination. Accordingly, in preparing for transfusions hospital laboratories usually group the donors' cells by observing them in certified strongly agglutinating sera types A, B, and O. Then major and minor cross matchings are made with donors and recipient of the same group. Thus the action of the donors' and recipient's sera or incompatible cells is not ordinarily observed.

Moss[1] wrote in 1910, referring to hemolysis, "In regard to the relationship existing between isoagglutinins and isohemolysins, I may say that agglutination frequently occurs independently of hemolysis but that the inverse relation occurs, i.e., hemolysis without the simultaneous or preceding occurrence of agglutination seems less likely.

"As a rule, however, agglutination proceeds more rapidly than does hemolysis, and by observing the action of a serum, which contains both agglutinin and hemolysin, on susceptible corpuscles, one frequently sees agglutination set in which is subsequently broken up as hemolysis takes place; so that if the observations are not made until the lapse of 2 hours, in a case where the hemolysin is not quite sufficient to dissolve completely all of the corpuscles present, we may get the appearance of hemolysis having taken place without agglutination."

In 1927 J. Buren Sidbury[2] remarked that, ". . . too little emphasis has been laid on hemolysis in blood matching . . . hemolysis is forgotten about in the usual routine of blood matching, since it occurs so infrequently." He observed hemolysis without simultaneous or preceding agglutination 10 times in two years while matching bloods for transfusions.

According to Stitt,[3] agglutinins and hemolysins are separable by heat, and hemolysis is not necessarily preceded by agglutination.

For the convenience of readers the different classifications for the blood groups are tabulated. In the Landsteiner or International classification the

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capital letters represent inherited dominant agglutinogens of cells. Corresponding active agglutinins (or hemolysins) of sera are indicated by the small Greek letters (Bernstein[4]).

Jansky classification	IV	II	III	I
Moss classification	I	II	III	IV
Landsteiner classification	AB	A	B	O
Active agglutinins	–	β	α	$\alpha\beta$

Cell suspensions of customary density were obtained for the blood grouping tests, described herein, by mixing a drop of fresh blood with about 3 c.c. of a solution of 1 per cent sodium citrate in physiologic saline. A drop of the suspension was mixed with a drop of the serum to be tested on a cover glass and inverted over the vaseline-rimmed hollow of a hanging-drop slide. The preparations were rocked back and forth several times to insure mixing before being examined under the microscope. Since all sera were tested before chilling, although some were also tested after being in the refrigerator, we are not here concerned with "cold agglutinins" or "cold hemolysins."

At this point it may be well to describe the degree of hemolysis ordinarily encountered. The effect of 8 sera from known group O individuals on known group A cells from four sources were studied. Agglutination started in all preparations in from one-half to two minutes and was moderate or marked in one and one-half to three minutes. There was no hemolysis in 6, and only the ordinary slow hemolysis in 26 of the 32 preparations. The hemolysis usually began in three to ten minutes, but sometimes not until after one-half hour. It was usually very slow, but in some cases it was moderate. In all preparations the microscopic field was filled with cells after one-half hour and there were many clumps, usually heavy and fused but sometimes loosened. The picture was usually the same after one or one and one-half hours longer, but in a few instances moderate hemolysis dissolved enough of the cells so that there were as few as 40 cells after one and three-quarter hours. The number of cells remaining after a specified time seemed to depend on the density of the cell suspension and the amount of serum used.

When these 8 known group O sera were similarly tested with group AB cells from two sources, agglutination was moderate or marked in two minutes, and slow hemolysis, present in all preparations, began in five to ten minutes. Here again all fields were crowded or moderately filled with cells and showed heavy clumps after one-half hour.

Three instances of very rapid specific hemolytic action with very little or no agglutination by sera of group O patients, who had received sulfanilamide therapy for beta hemolytic streptococcus infection and low grade bacteriemia, are presented.

CASE 1.—A 22-year-old group O male attendant was diagnosed as having a beta-hemolytic streptococcus bacteriemia and treated with very high dosages of sulfanilamide.

Culture of exudate from an infected finger on the day of admission was positive for beta hemolytic streptococci; blood culture showed gram-positive cocci in chains one hour after inoculation of the broth but it was negative after twenty-four hours. Subsequent blood cultures on the seventh and eighth days were negative.

Daily sulfanilamide therapy was begun on the day of admission. The blood level for free sulfanilamide increased from 4.8 mg. per cent on the seventh day to 28.4* mg. on the eleventh day, and then decreased to 6.7 mg. on the seventeenth day. It was 2.3 mg. on the eighteenth day, at which time it was discontinued.

On the eighth day the blood sugar was 148 mg. per 100 c.c. and the nonprotein nitrogen was 43 mg. per 100 c.c. On the eleventh day the nonprotein nitrogen was 45 mg. per 100 c.c.

The white blood cell count rose from 9,400 per c.mm. on the day of admission to 23,900 on the fourteenth day, and then decreased to 5,500 by the nineteenth day, staying at this level until the thirtieth day when it was 7,600. The red blood cell count decreased from 4,010,000 per c.mm. on the day of admission to 3,280,000 on the fourteenth day and 2,360,000 on the nineteenth day, then it increased steadily to 4,090,000 by the thirtieth day.

The urine showed a slight trace of albumin and a slightest possible trace of sugar on the day of admission. Subsequently no sugar was found. The albumin varied from negative to trace or heavy trace, at which times a rare hyaline or finely granular cast was found in the sediment. A slightly positive foam test for bile was noted on the eighth day only. Urinalyses on the thirtieth and thirty-second days were essentially negative.

No transfusions were given.

The patient was dismissed from the hospital ward on the thirty-first day.

On the eighth day of the patient's illness, while he was receiving sulfanilamide therapy for his streptococcic infection, a preliminary determination of his blood group was made by the method of major and minor cross-matchings, with known group A cells and serum. In five minutes the group A cells which had been mixed with the patient's serum were not to be found. Then it was observed that not only did this serum hemolyze group A cells very rapidly but it did this without any observable agglutination. Under the microscope the individual cells were seen to diffuse, shrink, and disappear in about five minutes. Since, in addition, in the minor matching the group A serum did not agglutinate nor hemolyze the patient's cells, his blood was classified as type O. This grouping was confirmed on the seventeenth day by the non-agglutination of the patient's cells in certified typing sera A, B, and O.

The blood grouping properties were studied further eighty-nine days after the first day of illness which was seventy-one days after discontinuance of sulfanilamide therapy. The patient's serum again rapidly hemolyzed without noticeable agglutination known group A cells from two sources, hemolysis beginning before two minutes and practically complete in five minutes. It similarly hemolyzed known group AB cells from two sources in five minutes. However, Group B cells from two sources were not hemolyzed. Instead the usual rapid agglutination took place, beginning before two minutes and marked in five to six minutes.

Similar blood group characteristics were observed on the ninety-eighth day, eighty days after cessation of sulfanilamide therapy. The patient's cells were again tested in certified sera of types A and B. Since no agglutination nor hemolysis took place during one hour, the blood was again found to belong to group O. The patient's serum still hemolyzed known AB cells from

*Dr. K. V. Quinn wishes to point out that at one time in this illness, while the patient was receiving the excessively high dosage of 240 grains of sulfanilamide daily for four days, the blood level reached 28.4 mg. for two days. On reducing the dosage, the blood level fell steadily with no unfavorable results except for the 2,360,000 red blood cell count.

two sources and known A cells from two sources without agglutination, the hemolysis beginning in one minute and complete in five minutes, except for one cell left in an originally crowded field. As before there was no hemolysis of known group B cells from two sources even after thirty to forty minutes, the usual agglutination beginning in one minute and marked in two to four minutes. There was no evidence of agglutination or hemolysis of known group O cells from two sources during eighty minutes.

One month later, on the one hundred and twenty-sixth day, about three and one-half months after the sulfanilamide had been discontinued, the hemolytic action was still marked but tended more toward normal. In one to two minutes there was a slight tendency to agglutinate in 2's and 3's group AB cells from two sources and group A cells from four sources, but these clumps were soon loosened and broken up by the fairly rapid hemolysis. This began in about three minutes and finally left in the microscopic field only from 10 to 100 AB cells in twenty minutes and from 0 to 200 A cells in six to twenty minutes. The effectiveness of the hemolysis now seemed to be more dependent on the density of the cell suspension, the proportion of serum, and the source of the cells. As before, the normal agglutination of group B cells from two individuals took place without hemolysis. There was no agglutination or hemolysis of known group O cells from two individuals during one hundred minutes.

CASE 2.—The second patient was a group O female undifferentiated high grade moron, 23 years of age, suffering from a beta hemolytic streptococcic infection of the knee and a low grade bacteriemia treated with sulfanilamide.

Two days after admission blood culture showed gram-positive cocci in pairs. Operation was performed on the seventh day. Cultures from the wound were positive for beta hemolytic streptococci. On the eighth day blood culture was positive for gram-positive cocci in pairs and short chains. Another operation was performed on the twenty-third day. Blood cultures taken on the twenty-third and forty-fifth days were negative.

Sulfanilamide therapy was started on the twenty-fourth day. The blood sulfanilamide level was 5.4 mg. per 100 c.c. on the twenty-fifth day and increased by the thirty-first day to 12.3 mg. where it remained for three days. It fell to 7.4 mg. on the thirty-seventh day, but increased again to 12.5 mg. by the fortieth day. On the forty-first day, when sulfanilamide treatment was discontinued, the blood level was 9.7 mg. per 100 c.c. No sulfanilamide was found in the blood on the forty-fourth day.

The blood sugar was 140 mg. per 100 c.c. on the evening of admission. On the third day the fasting blood sugar was 127 mg. per 100 c.c. On the eighth day the fasting blood sugar was 110 mg. per 100 c.c. and the nonprotein nitrogen was 31 mg. per 100 c.c.

The urine showed a slight trace of albumin and a slightest possible trace of sugar the morning after admission. Thereafter the sugar was negative and the albumin was either negative or slightly positive. On the eighth day an occasional hyaline or coarsely granular cast was found in the sediment and the foam test for bile was slightly positive.

The remaining clinical data seemed irrelevant, and since they will probably be published in another connection, they are omitted here. The patient was still convalescing on the one hundred and second day.

On the twenty-fifth day, one day after sulfanilamide therapy for the streptococcus infection had been started, the patient's blood was typed for transfusion. Since her cells were not clumped in certified sera of groups A, B, and

O, she belonged to the O group. Similarly, her mother and her stepfather were found to belong to the O group. Major and minor cross-matchings of the blood of these donors with that of the patient showed complete compatibility, only harmless, slight but definite rouleau formation being observed in repeated preparations of the patient's serum and the donors' cells. On this twenty-fifth day transfusion was done using the mother's blood with favorable results. Blood from the stepfather was also taken and stored.

As a matter of interest the patient's serum before transfusion was matched with known group A cells. Surprisingly, in this case too there was observed no agglutination but rapid hemolysis complete in a few minutes.

On the thirtieth day the patient's serum was again matched with the cells of the stepfather's preserved citrated blood of five days before, and as before there was only definite rouleau formation. Satisfactory transfusion was done using this blood.

On the forty-fourth day fresh blood from the mother was found to be compatible with the patient's blood and another transfusion was given using this donor.

On the sixty-third day, twenty-two days after the sulfanilamide was discontinued the blood grouping properties were again studied. The patient's serum rapidly hemolyzed known group A cells in seven to ten minutes without apparent agglutination. It also hemolyzed group AB cells in two to seven minutes without agglutination. It agglutinated group B cells in the usual fashion, however, no hemolysis being observed during twenty minutes.

Blood grouping characteristics were observed further on the seventy-third day. As before, the patient's cells were neither clumped nor hemolyzed in certified group A and Group B sera, again indicating that she belonged to the O group. Her serum hemolyzed known group AB cells from two sources and known group A cells from two sources without agglutination, hemolysis starting in one minute and practically complete in four to seven minutes. Again this serum agglutinated known group B cells in the usual fashion, beginning in one to two minutes and becoming marked in three minutes. This time, however, the ordinary slow type of hemolysis of the group B cells was seen to accompany the ordinary agglutination. It began in about two to three minutes and continued so slowly that after one-half hour there were still clumps and about 500 cells in the field; after one and three-fourths hours there were clumps and about 100 cells; after three hours about 15 single cells remained. This was evidently the common type of hemolysis due to the beta hemolysin and very different from the rapid hemolytic action of the group AB and A cells.

One month later, i.e., on the one hundred and second day of illness and two months after the sulfanilamide had been discontinued, the blood group characteristics tended a little more toward normal. Now there was slight to moderate clumping of AB cells from two sources in one to one and one-half minutes, but hemolysis began in two and one-half minutes, disintegrating the clumps and dissolving the cells so that in twenty minutes only 2 to 10 cells

were left in a field originally crowded. Similarly, when the serum was matched with group A cells from four sources there was a very slight to moderate clumping in one-half to one minute, but hemolysis started in one and one-half to two minutes, loosening the clumps and destroying the cells so that in eleven to seventeen minutes only 1 to 5 cells remained in the field. As before there was the usual agglutination of group B cells from two sources, this time with practically no hemolysis. There was neither hemolysis nor agglutination of known group O cells from two individuals.

CASE 3.—A third case is that of a 14-year-old group O female familial low-grade moron. Three days before admission she had been confined to bed with an acute follicular tonsillitis. On the day of admission, the fourth day of her illness, her axilla temperature rose to 103.8° F. and she had severe chest pain. She was diagnosed as having a beginning streptococcus pneumonia and she was treated with sulfanilamide.

Blood culture, taken on the seventh day of illness, was positive after seventy-two hours for rare gram-positive cocci occurring singly and in pairs.

Sulfanilamide therapy was started about 12:01 A.M. of the fifth day and discontinued the thirteenth day. The blood levels were 11.8, 9.9, 12.5, 11.1, 4.7, 6.1, 4.6, 3.2, 1.5, and 0.5 mg. per 100 c.c. from the fifth through the fourteenth days, respectively.

The red blood cell count varied during the illness between 4,260,000 and 5,270,000 per c.mm. The white blood cell count was 13,650 per c.mm. on the fourth day and rose to 17,500 on the eleventh day but decreased to 7,800 by the fourteenth day. The urine was essentially negative except for a very slight trace of albumin.

The patient showed steady improvement under treatment and was discharged on the nineteenth day.

In this case the illness was not so grave and the sulfanilamide therapy was not so long continued as in the previous 2 cases. Nevertheless, a definitely increased rate of hemolysis of group AB and group A cells by this patient's serum was also seen.

Her blood group characteristics were studied on the fifth day of illness and only 15 hours after the first dose of sulfanilamide. The cells, observed during two and one-half hours, did not clump in certified sera types A, B, and O, and the patient, therefore, belonged to group O. Although her serum began a moderate agglutination of known group A cells in one minute, rapid hemolysis started in one to two minutes, disintegrating the clumps and completely hemolyzing in four to five minutes all the cells in a previously crowded field. Similarly, this serum began a moderate agglutination of group AB cells from two sources in one minute, but rapid hemolysis started in one and one-half to two minutes, disintegrating the clumps and completely hemolyzing in four to six minutes all the cells except one in a previously crowded field. Matching with known group B cells from two sources resulted in the usual agglutination, beginning before one minute and marked in two to three minutes, without noticeable hemolysis.

Four days later, on the ninth day of illness and after five days of sulfanilamide, her serum showed a slight agglutination of AB cells in one-half minute but hemolysis began in one to two minutes disintegrating the clumps and dissolving the cells so that in six to fourteen minutes there were only 1 to 20 cells left in the microscopic field. The number of AB cells remaining ap-

parently depended somewhat on the density of the cell suspension and the amount of mixing motion given to the preparations. This serum also started to agglutinate known group A cells from one individual in one to one and one-half minutes but rapid hemolysis set in in one and one-half minutes, disintegrating the clumps and dissolving the cells so that after six to fifteen minutes only 1 to 7 cells remained in the field. There was only a very slight tendency to clump A cells from another individual, rapid hemolysis dissolving all the cells in three minutes. The action on group B cells from two sources was the usual agglutination without hemolysis. On group O cells from two sources there was no effect except for slight rouleau formation.

On the twenty-second day, nine days after discontinuance of the sulfanilamide, the hemolysis was still somewhat rapid. The serum began a slight to moderate clumping of AB cells from two sources in one-half minute, but hemolysis started in two minutes, broke up the clumps, and left only 2 to 7 cells in the field after nine to fifteen minutes. However, with an exceptionally heavy cell suspension, about 50 cells remained, showing that the hemolysis was neither so strong nor so rapid as to be independent of the number of AB cells. The action of this serum on known A cells was similar. In one-half to one minute there began a slight tendency to clumping which became more marked in the succeeding minute. However, rapid hemolysis set in in about two minutes, breaking up the clumps and dissolving the A cells so that in six to thirteen minutes only 1 to 6 cells remained in previously crowded fields. As before, group B cells from two sources were clumped at once without evidence of hemolysis. There was neither agglutination nor hemolysis of group O cells from two sources during one and one-half hours.

DISCUSSION

This rapid hemolysis by sera of group O patients having beta hemolytic streptococcus infection treated with sulfanilamide is evidently very different in degree at least from the ordinary two-hour hemolysis mentioned by Moss. Inasmuch as it is specific for cells containing the A agglutinogen, since only group AB and group A cells are thus hemolyzed, it is probably associated with the alpha agglutinin of the serum. In this connection it is of interest that the serum of a group A patient who had been treated with sulfanilamide showed no unusual blood grouping properties. This is consistent with the foregoing, since the alpha agglutinin in group A serum is inactive.

Interpretation is, of course, impossible with only 2 or 3 cases. Unfortunately, blood grouping tests were not made before the administration of sulfanilamide. However, the definite though temporary agglutination preceding the somewhat less rapid and less complete hemolysis, which was observed in the third case and also in the other two cases two or three months after cessation of sulfanilamide therapy, suggests that the rapid hemolytic action is probably not normal for these individuals. Moreover, it is probably not due to the infection alone, since then the phenomenon would have been noticed long ago. A glance through recent literature revealed no mention

of such increased group-specific hemolytic action of serum due to infection. Wünsche[5] did not mention it in a paper on the relation between the blood group and the hemolytic power of streptococci. Therefore, it seems more logical to assume that the effect is due to the action of sulfanilamide on the alpha hemolysin.

For some time workers have sensed that there might be some connection between the blood groups and susceptibility or immunity to disease. Alexander[6] thought that the tendency to malignant disease depended on the blood group, but this was denied.[7]

A few published observations point to an immunologic peculiarity of the alpha agglutinin. Straszynski[8] in 1925 found that the Wassermann reaction was related to the blood group, group AB having 2.4 times as many positive Wassermann reactions as group O. He concluded that the rapidity with which the Wassermann reaction disappears under the influence of antisyphilitic treatment is a constitutional property existing in correlation with the blood group.

Hirszfeld, Hirszfeld, and Brokman[9] found that the susceptibility to diphtheria, as indicated by the Schick test, is inherited conjointly with the blood properties and must also depend on constitutional characters. They stated that infection or immunization can mobilize unrevealed immunologic forces and can accelerate the process of changing from Schick positive to Schick negative, but that this is dependent on the constitutional inherited capacity of the individual.

Balgairies and Christiaens[10, 11] found a remarkable elevation of agglutinin anti A after injection of diphtheria antitoxin and of tetanus antitoxin, which diminished progressively to a stable level in six months and which they attributed to the presence in the antitoxin of a substance having the same nature as agglutinogen A.

An analogous explanation for the cases described herein would be that sulfanilamide is related to agglutinogen A and that in the body it stimulates formation of the anti A or alpha hemolysin. However, the action may be more indirect through an influence on the liver, kidney, or bone marrow, etc.; or, since with physiologic saline there is said to be potentiation[12] of the bactericidal effect of this drug, perhaps its action is catalytic. It may thus upset a balance of hemolytic-agglutinative activity in favor of the hemolysis. In this connection it is of interest that in Cases 1 and 2, when hemolysis without agglutination was substituted for agglutination in the scheme for determining the blood group, the result agreed with that found when only the cells were tested in certified sera. This supports a theory of the interdependent action of hemolysin and agglutinin.

Further study of the facts relating to this hemolysis may eventually lead to interesting practical and theoretical conclusions. For instance, such a study may help us to understand and to prevent certain unfavorable transfusion reactions; it may explain the curative action of sulfanilamide as well as its now unpredictable toxicity for some patients and its inefficacy for others; it may give us a clearer picture of the mechanisms underlying the

action not only of sulfanilamide and perhaps of antisyphilitics and other chemicals, but even of immunizing preparations. However, the aim of this paper is neither to add to the list[13] of conjectures as to the mode of action of sulfanilamide nor to interpret the findings presented. It is merely intended (1) to record the facts of rapid group-specific (anti A) hemolysis with little or no agglutination by sera of three group O patients having beta hemolytic streptococcus infection treated with sulfanilamide; (2) to point out the possible theoretical and practical importance of these facts; and (3) to request that physicians in general hospitals will likewise report the effect of sera of their patients, especially of group B and group O patients, on cells of the different groups before, during, and after sulfanilamide therapy.

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VITAMIN A DEFICIENCY IN DISEASES OF THE LIVER: ITS DETECTION BY DARK-ADAPTATION METHOD*

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THERE is considerable evidence that the liver plays an important role in regulating the concentration of vitamin A throughout the body. We are indebted to Moore[1] for stressing this point and for demonstrating the liver to be the chief depot for vitamin A storage in man. This has also been shown experimentally by Sherman and Boynton.[2]

The experimental work of Simonnet and Bousson[3] and Olcott and McCann[4] would indicate that the conversion of carotene, the precursor of vitamin A, is effected in the liver, presumably, by an enzyme called carotenase. However, this conversion in man is not essential when vitamin A is provided in the diet.

That the metabolism of vitamin A is linked with the hepatic system may be illustrated by the occurrence of signs of avitaminosis A in some patients with prolonged jaundice, despite the fact that their diets are quite adequate in vitamin A content. Thus, Altschule[5] found microscopic evidence of vitamin A deficiency in 6 out of 11 patients suffering with severe and protracted jaundice due to congenital atresia of bile ducts. The association between hepatic disease with jaundice and vitamin A deficiency is further illustrated by the observations of Blackfan and Wolbach[6] and Owen and Hennessey.[7] They found epithelial metaplasia in certain tissues in spite of the fact that the patients received an amount of vitamin A in their diets considered sufficient for the protective needs of the body. It is probable that the manifestations of avitaminosis A were conditioned by defective utilization, storage, or absorption of vitamin A, or its precursor, carotene.

When the parenchyma of the liver is injured, a disturbance in its function is to be expected. Early clinical features of vitamin A deficiency in such pathologic states may be overlooked; they occur irregularly and inconstantly, before definite ocular changes develop. The eye changes (xerophthalmia, etc.), as is generally held, occur in the advanced stages of the disease.

The early recognition of vitamin A deficiency in the course of liver disease assumes practical importance, particularly since parenteral administration of vitamin A has become available recently. The liver is known to be instrumental in the formation and storage of the other vitamins, particularly B_1 and B_2 complex, and vitamins C and D. It is also claimed that the presence of vitamin A in the liver enhances glycogen storage.[8]

The purpose of this investigation was to determine the early occurrence of avitaminosis A in patients with hepatic disease, measured by dark adaptation

*From the Philadelphia General Hospital and the Temple University Hospital.
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TABLE I

SUMMARY OF FINDINGS IN CASES WITH LIVER DISEASE

NO.	NAME	AGE	SEX	DURATION OF DISEASE	DARK-ADAPTATION THRESHOLD IN MICRO-PHOTONS	DIAGNOSIS	SIGNIFICANT LABORATORY FINDINGS	REMARKS
1	W. A.	64	M	2 years	1,691	Laënnec cirrhosis	Sugar, 91; urea N, 9; choles., 90; ester, 50; tot. pro., 6.5; alb., 1.8; glob., 4.7; ict. index, 10; Kahn, neg.	Paracentesis—5 liters and 12 oz. of clear, yellowish fluid removed. Liver palpable halfway to umbilicus
2	R. B.	42	M	10 days	636	Hepatocellular jaundice	Sugar, 70; urea N, 10; choles., 144; ester, 19%; tot. pro., 4.7; alb., 3.2; glob., 2.1; ict. index, 125. Bromsul., 37% retention. Takata-Ara strongly pos.	Three days after receiving neosalvarsan developed jaundice and epigastric pain
3	S. B.	43	M	14 months	546	Syphilitic cirrhosis	Sugar, 61; urea N, 16; choles., 148; ester, 65 (38%); ict. index, 13. Wass. +4	Exploratory laparotomy done. Syphilitic cirrhosis (hepar. lobatum)
4	H. B.	48	M	18 months	546	Laënnec cirrhosis	Sugar, 107; urea N, 12; choles., 142; ester, 51%; tot. pro., 5.7; alb., 2.1; glob., 3.6; ict. index, 20. Takata-Ara strongly pos. Bromsul., 25% ret.	Patient heavy drinker. Seventeen months ago had Talma operation. Felt well until present admission. Twelve paracenteses. Patient died with hemorrhage from bowel
5	R. C.	23	M	1 month	Normal	Hepatocellular jaundice	Sugar, 107; urea N, 10; N.P.N., 36; choles., 141; ester, 81 (55%); tot. pro., 8.3; alb., 4.5; glob., 3.8. Takata-Ara pos. Ict. index, 23; Wass. neg.	Jaundice developed during stay in hospital. Previous history of gall bladder. Jaundice cleared on discharge from hospital

6	W. G.	25	M	1 month	1,025	Syphilitic hepatitis	Sugar, 72; urea N, 9; choles., 320; ester, 87 (22%); tot. pro., 7.1; alb., 3.7; glob., 3.4; ict. index, 72. Takata-Ara neg; phosphatase, 10; Wass. +4	Liver 10 cm. below costal margin. Bismuth and iodides caused decrease in size of liver to 3 cm. Jaundice cleared
7	W. G.	37	M	3 weeks	Normal	Hepatocellular jaundice (catarrhal)	Sugar, 87; urea N, 11; ict. index, 20. Wass. neg.	Jaundice following attack of pneumonia
8	J. B.	50	M	5 years	19,600	Syphilitic cirrhosis of the liver	Sugar, 63; urea N, 22; choles., 192; ester, 91 (47%); tot. pro., 7.5; alb., 3.2; glob., 4.3; ict. index, 10. Bromsul., 24% retention. Wass. +4	Numerous paracenteses. Antisyphilitic treatment, 2 c.c. bismuth and mercupurin. Ascites under control 3 weeks prior to admission to hospital when ascites recurred; 2½ liters clear yellow fluid obtained. Liver edge 5-6 cm. below costal border. No recurrence of ascites. Is under antisyphilitic treatment. Has also aortic valvulitis
9	V. D.	48	M	2 months	1,007	Atrophic cirrhosis	Sugar, 103; urea N, 13; choles., 156; ester, 53%; tot. pro., 6.5; alb., 2.3; glob., 4.4; Takata-Ara strongly positive. Wass. neg.	Patracentesis yielded 5¼ liters of yellow fluid. Liver normal size. Spleen palpable to umbilicus. Drank 4-5 glasses wine daily for several years
10	W. K.	56	M	3 weeks	*1,190 1,130 1,434	Atrophic cirrhosis of the liver	Sugar, 65; choles., 78; ester, 54%; tot. pro., 6.0; alb., 2.1; glob., 3.9. Bromsul., 28% retention	Consumed a pint of whiskey daily for 5 months. Vomited blood. Distended abdominal veins. Ascites. Four months later patient died of pneumonia
11	M. L.	46	F	1 year	309	Carcinoma of the gall bladder with metastasis to the liver	Sug., 81; urea N, 9; choles., 309; tot. pro., 6.9; alb., 4.2; glob., 2.7; Takata-Ara strongly pos.; ict. index, 85	Exploratory laparotomy revealed carcinoma of gall bladder with metastasis to the liver
12	A. M.	20	F	14 days	408	Hepatocellular jaundice following neosalvarsan	Sugar, 59; urea N, 8; choles., 176; ester, 81 (46%); ict. index, 42. Wass. +4	Patient delivered of healthy baby. Had granuloma inguinale. Given tartar emetic every 2 days in increasing amounts. On 3rd day received 5 c.c. After receiving neosalvarsan and tartar emetic, she developed jaundice and fever. General condition improved on discharge from hospital, but still jaundiced

*The thirty-minute thresholds taken at different times.

TABLE I—CONT'D

NO.	NAME	AGE	SEX	DURATION OF DISEASE	DARK-ADAPTATION THRESHOLD IN MICRO-PHOTONS	DIAGNOSIS	SIGNIFICANT LABORATORY FINDINGS	REMARKS
13	R. M.	62	F	2 years	204	Atrophic cirrhosis with jaundice	Choles., 276; ester, 40%; ict. index, 34	Patient consumed ½ pint of whiskey daily for 25 years. Liver 4 fingerbreadths below costal margin
14	R. M.	35	M	3 months	Normal	Atrophic cirrhosis	Choles., 170; ester, 102 (54%); tot. pro., 5.4; alb., 2.3; glob., 3.1; ict. index, 9. Blood vitamin C 0.36 mg.	Two paracenteses. Liver 5 cm. below costal margin. Heavy drinker
15	P. R.	54	M	4 months	273	Cirrhosis of the liver	Sugar, 96; urea N, 26; choles., 108; ester, 66 (61%); tot. pro., 5.2; alb., 2.0; glob., 3.0; ict. index, 8	Paracentesis yielded 2½ gallons of clear yellowish fluid
16	J. D.	44	M	8 months	233	Primary carcinoma of the liver	Sugar, 100; urea N, 12; tot. pro., 5.8; alb., 2.9; glob., 2.9; choles., 224; ester, 120 (53%). Bromsul., 8% retention	Marked ascites. Liver hard and nodular
17	H. D.	17	F	2 months	Normal	Acute catarrhal jaundice	Sugar, 98; urea N, 8; choles., 126; ester, 63 (50%); ict. index, 60	Left hospital free from symptoms except slight jaundice which is clearing
18	Z. H.	33	M	2 months	*572 694 744 1,309	Hepatocellular jaundice	Sugar, 68; urea N, 9; ester, 50; tot. pro., 6.1; choles., 504; alb., 2.8; glob., 3.3; ict. index, 110. Takara-ara strongly pos.	Jaundice following injections of neosalvarsan. Cholesterol kept rising to 1,490 mg. with 2 % ester.

19	E. M.	54	M	2 months	240	Cirrhosis following hepatocellular jaundice	Sugar, 79; urea N, 12; choles., 194; tot. pro., 7; alb., 3.9; glob., 3.1; ester, 105 (53%). Takata-Ara strongly pos. Bromsul., 24% ret.; ict. index, 12	Jaundice cleared after 9 weeks' hospitalization. Liver 3 cm. below right costal margin with tenderness in the right upper quadrant
20	E. G.	29	F	10 years	299	Biliary cirrhosis	Sugar, 6.8; urea N, 10; choles., 370; ester, 192 (52%); tot. pro., 6.8; alb., 3.9; glob., 2.9; bromsul., 40% retention; ict. index, 43. Takata-Ara neg.	Tenderness and rigidity in right upper quadrant. Liver and spleen enlarged and tender. Poorly functioning gall bladder (x-ray). First gall bladder episode 10 years ago. Recurrent attacks of jaundice with gastrointestinal symptoms
21	G. R.	53	M	3 weeks	390	Laënnec cirrhosis	Sugar fasting, 82; choles., 300; ester, 56%; tot. pro., 6.5; alb., 2.9; glob., 3.6. Bromsul., 40% retention. Ict. index, 115; Wass. negative. Phosphatase, 19 units	Patient drank couple of beers and whiskeys daily since 1909. Liver edge felt 5 fingerbreadths below right costal edge. Edge smooth and movable. Three paracenteses averaging 3,600 c.c. Patient died in hepatic coma
22	A. C.	68	M	11 days	285	Hepatocellular jaundice	Sugar, 98; choles., 218; ester, 108; urea N, 17; ict. index, 46. Wass. neg.	Jaundice following acute sinusitis. Patient remained in hospital 18 days. Jaundice disappeared when he left hospital
23	D. S.	70	M	2 years	28,625	Carcinoma of the the liver	Ict. index, 97; blood Wass., +4. Van den Bergh direct strongly pos.	Diagnosis of primary carcinoma of liver confirmed by post-mortem examination of tissue
24	W. C.	58	M	3 years	19,670	Primary carcinoma of the liver	Blood sugar, 75; urea N, 18; choles., 240; ict. index, 17; Wass. neg.	Paracentesis—2,300 c.c. bloody fluid obtained. Diagnosis confirmed by post-mortem examination. Ascitic fluid 300 c.c. on 2/20/39, 3 days before death
25	A. R.	72	M	2 months	177	Calculus in the common duct	Blood sugar, 87; urea N, 12; ict. index, 80	Jaundice progressively worse for past 2 months. X-ray of stomach and intestines negative

under controlled conditions. We selected the dark-adaptation method because evidence has been presented by various investigators that poor dark adaptation is directly related to vitamin A deficiency.[9]

The effect of vitamin A therapy on the progress of the illness, as well as on the hepatic function, is under investigation and will be described elsewhere.

Dark Adaptation.—"Dark adaptation" is the ability of the eye to adapt itself to darkness. An example of this is the entering into a darkened cinema theater after walking in the street on a sunny day. At first it is impossible to see the seats in the theater due to the fact that the visual purple was bleached out by the sun. After a specific interval, the objects and people within the theater appear somewhat clearer. As time goes on, this improvement in vision progresses until the vision becomes as clear as if the theater were illuminated. This physiologic phenomenon is accomplished through the regeneration of the visual purple and depends, in the main, upon its vitamin A content.

Pathologic dark adaptation is divided[10] into two types: (1) organic and (2) functional. The *organic type* is the result of deep retinal or choroidal pathology and is recognized by ophthalmoscopic examination. It is noted in such ocular diseases as retinitis pigmentosa, choroiditis, etc. In the absence of retinal, choroidal, or other ocular pathology, a delay in regeneration of visual purple above the normal and specified time is called a *functional or latent* pathologic dark adaptation. This latter condition is attributed to an avitaminosis A.

Technique.—All tests were carried out in a darkroom by the method described elsewhere,[11] with the American Optical Co. instrument devised by one of us (F.).

Material.—This presentation is based on a study of 25 persons. Careful history, physical examination, and ophthalmologic and laboratory studies were made on each patient. The patients were unselected and represented various types of liver disease as studied in the wards of the Philadelphia General Hospital and the Temple University Hospital during 1938 and 1939.

RESULTS

A summary of the significant findings in these patients is given in Table I.

Thirteen patients with cirrhosis of the liver were examined. Six patients in this group gave a history of alcoholism, and 3 with a plus 4 Wassermann had a history of syphilis.

Patient E. G. in Table I represents the only case of biliary cirrhosis in our series. Her hepatic symptoms were of ten years' duration and were secondary to a chronic cholecystitis. The liver function tests showed moderate hepatic damage.

In patients with Laënnec cirrhosis, the livers were hard and palpable, with ascites in all cases. The liver function tests, as shown in Table I, column 7, displayed varying degrees of liver damage. Jaundice was absent in 4 patients. Seven patients showed jaundice, varying from a slight icteric tinge of the sclerae (icterus index 13) to a diffuse jaundice of the body (icterus index 115). In 2 patients the icterus index was not determined.

While it is fully realized that an absolute diagnosis of cirrhosis of the liver can be made during life only by biopsy, in this group the history and the

course of the disease, coupled with the laboratory studies and clinical data, aided in confirming the diagnosis. In 4 cases the diagnosis was corroborated by autopsy.

All patients with cirrhosis of the liver, including the syphilitic type, showed degrees of pathologic dark adaptation, varying from 204 to 19,600 microphoton.* Three patients complained of night blindness, determined after careful questioning.

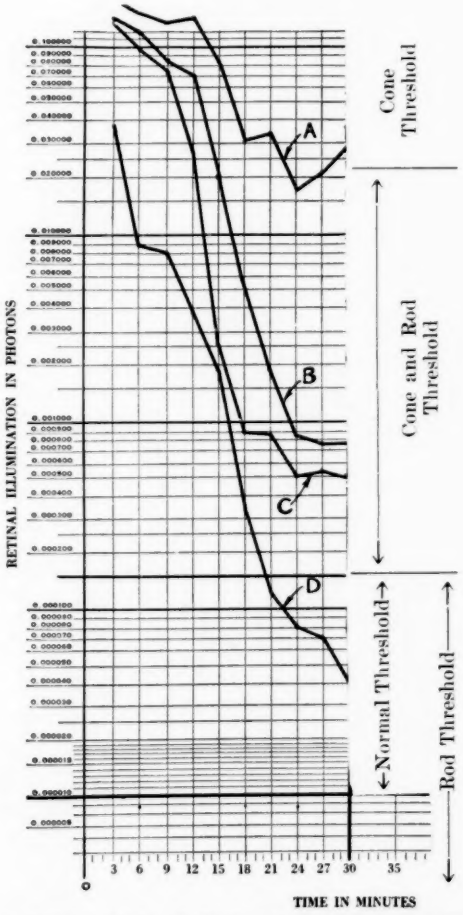

Fig. 1.—Illustrating various types of dark adaptation curves in a series of cases. *A.* Case 23, Table I, carcinoma of the liver. *B.* Case 1, Table II, delirium tremens and neuritis. *C.* Case 4, Table I, hepatocellular jaundice (neoarsphenamine). *D.* Case 17, Table I, hepatocellular jaundice (catarrhal).

It is interesting in this connection to note that Patek[12] and Snell[13] have reported clinical improvement in individuals with cirrhosis of the liver following the conventional treatment combined with vitamin therapy.

Pertinent to this discussion is the recent study by Connor,[14] in which he maintains that it is very doubtful whether a person consuming a balanced diet in addition to an excessive amount of alcohol would develop alcoholic cirrhosis.

*Normal dark-adaptation threshold is up to 150 microphotons within thirty minutes.

TABLE II

SIGNIFICANT FINDINGS IN ALCOHOLIC ADDICTS

NO.	NAME	AGE	SEX	DURATION OF DISEASE	DARK-ADAPTATION THRESHOLD IN MICRO-PHOTONS	DIAGNOSIS	LABORATORY FINDINGS	REMARKS
1	F. B.	26	M	4 weeks	766	Incipient delirium tremens and neuritis of lower extremities	Blood sugar 87. Urea N 11. Kahn negative	Addicted to alcohol since 1931. Consumed 1 quart alcohol daily for 4 weeks. Had no other food but soup and tea. Liver and spleen not palpable
2	T. M.	59	M		701	Alcoholic psychosis and cerebral arteriosclerosis	Blood sugar 68. Urea N 10. Kahn negative	Chronic alcoholic. Lives alone. Drinks 1 or 2 jiggers of whiskey a day and some beer. Liver and spleen not palpable. Diet soup and bread
3	F. C.	35	M	Several weeks	292	Delirium tremens (sore mouth and tongue)	Blood sugar 63. Urea N 11. Kahn negative	On admission patient presented sore mouth and tongue. He was on a high vitamin diet for 19 days prior to dark adaptation
4	F. D.	23	M	1 week	110	Delirium tremens	Blood sugar 82. Urea N 13. B.M.R –24%. Wassermann negative	Addicted to alcohol for 5 years. For 1 week prior to admission has eaten 2 bowls of soup and 2 cups of tea daily. Liver and spleen not palpable. Has colloid goiter. Patient was receiving desiccated thyroid, 1 grain daily. Is night blind
5	M. L.	42	M	Several weeks	196	Incipient delirium tremens	Blood sugar 87. Urea N 8. Kahn negative	Addicted to alcohol for 5 years. Liver and spleen not palpable
6	J. D.	34	M	4 months	339	Alcoholic psychosis	Blood sugar 79. Urea N 13	Addicted to alcohol for 9 years. Liver and spleen not palpable. Diet soup and bread
7	F. W.	46	M		204	Involutional state Chronic alcoholism	Blood sugar 82. Urea N 13. Wassermann negative	Dark adaptation 2 months after admission. Was receiving betaxin for 2 weeks prior to dark adaptation. Liver and spleen not palpable
8	P. M.	40	M		425	Alcoholic hallucination	Blood sugar 68. Urea N 9	Chronic alcoholic addict. Has been getting betaxin for 1 month prior to dark adaptation
9	P. O.	55	M	3 months	251	Alcoholic neuritis	Blood sugar 89. Urea N 9. Wassermann and Kahn negative	Addicted to alcohol for several years. Takes 3 drinks daily. Food consists of meat, bread, calves' liver one-half ounce weekly, milk one glass daily, spinach, carrots and potatoes small amounts
10	H. F.	49	M	Few days	196	Bromide psychosis Chronic alcoholic	Blood sugar 68. Urea N 10. Blood bromides 170. Wassermann negative	Chronic alcoholic; has consumed a pint of liquor a few days prior to drinking a 3 oz. medicine bottle containing bromide

In 10 patients addicted to alcoholism who were admitted to the psychopathic ward of the Philadelphia General Hospital, a study of their dietary regime prior to admission revealed a very poorly balanced diet, especially inadequate in vitamin A and vitamin B content. Dark-adaptation studies in this group showed that all except one had a marked pathologic curve (see Table II).

There were 7 patients with hepatocellular jaundice (see Table I), 3 of whom apparently utilized and stored their vitamin A, as evidenced by the normal rate of their regeneration of visual purple. The dark adaptation readings of these patients were, respectively, 42, 83, and 130 microphotons (normal up to 150 microphotons). Clinically these 3 patients manifested a minimal degree of liver damage, as evidenced by the clinical course of their illness and the liver function tests. Before their discharge from the hospital, the jaundice had entirely disappeared. A fourth patient belonging to this group had a slightly pathologic dark adaptation. When he left the hospital, his jaundice was clearing.

It is quite significant that in the remaining 3 patients there was marked pathologic dark adaptation: 408, 572, and 636 microphotons, respectively.* In each case the jaundice followed the administration of neoarsphenamine. The degree of jaundice was of the severe toxic type. Their clinical course was very stormy, and their various laboratory findings showed severe liver damage. When they were discharged from the hospital, there was still some evidence of jaundice. It is worthy of note that the dark adaptation pathology appeared to run parallel with the degree of liver damage conditioning the jaundice as gauged by the icterus index and other liver function tests. Thus, for example:

Case A. M., icterus index was 42, had a dark adaptation threshold of 408.
Case Z. H., icterus index was 110, had a dark adaptation threshold of 572.
Case R. B., icterus index was 125, had a dark adaptation threshold of 636.

We examined 4 patients with carcinoma of the liver, all of whom died. Their diagnosis was confirmed by the histologic examination of the tissues. They all presented pathologic dark adaptation to a marked degree (see Fig. 1, *A*).

One patient with advanced jaundice had a normal dark adaptation. The jaundice in this case was caused by a silent stone obstructing the common bile duct.

SUMMARY AND CONCLUSIONS

Twenty-five patients with various types of liver disease have been studied.

Ten patients with alcoholism, without clinical evidence of liver disease, were also examined.

Complete clinical laboratory and dark adaptation studies were made in each individual case.

*The light threshold in dark adaptation is the minimum light just visible to the dark adapted eye. Usually threshold readings are taken at three-minute intervals. Where the single threshold is given, it is the last or thirty-minute threshold, which in the normal case should be 150 microphotons.

A photon is a unit of retinal illumination and equals one candle per square meter per square millimeter of pupil area. A microphoton is one-millionth of a photon.

In 21 patients with liver disease the dark adaptation was pathologic. These patients, clinically and by laboratory studies, showed varying degrees of liver damage.

Of 7 patients with hepatocellular jaundice, 3 presented normal dark adaptation and minimal degree of liver damage. The remaining 4 patients showed evidence of liver damage and pathologic dark adaptation. In these 4 cases, the degree of liver damage, as evidenced by the icterus index and other laboratory studies, appeared to run parallel with the degree of dark adaptation pathology.

Nine out of 10 alcoholic addicts showed vitamin A deficiency as revealed by dark adaptation pathology. The tenth person, whose dark adaptation was normal, was receiving desiccated thyroid at the time the test was made.

We are indebted to Dr. M. M. Pearson for cooperation in the study of the alcohol addicts.

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1727 PINE STREET

THE SOURCES OF THE ENZYMES OF NORMAL AND PATHOLOGIC CEREBROSPINAL FLUID*

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INTRODUCTION

IN A PREVIOUS study[1] we found that normal cerebrospinal fluid contains small amounts of beta-glycerophosphatase and amylase, and occasional traces of lipase. In pathologic conditions of the central nervous system, such as purulent and tuberculous meningitis, hydrocephalus, brain tumor, brain abscess, and brain cyst, the cerebrospinal fluid was found to have increased enzymatic activity. The fluid of tuberculous meningitis was characterized by the presence of trypsin, antitrypsin, and tributyrinase, and by increased phosphatase, lipase, and amylase activity. The fluid of purulent meningitis was similar to that of tuberculous meningitis, except that trypsin and phosphatase were found in much greater amounts in the former. The cerebrospinal fluid of hydrocephalus, brain tumor, abscess, and cyst was found to have high tributyrinase and lipase activity. Antitrypsin and increased phosphatase were also found in some fluids of the brain lesion group.

Early in the course of our work the question of the source of the cerebrospinal fluid enzyme arose. We suggested, on theoretical grounds, that the enzymes of normal cerebrospinal fluid may result from the metabolism of brain tissue or may be derived from the enzymes of the blood plasma. The increased enzymatic activity of pathologic cerebrospinal fluid may be due to: (a) pleocytosis of the cerebrospinal fluid, (b) increased permeability of the meninges to plasma enzymes, and (c) destruction of brain tissue. We shall attempt here a discussion of the sources of the cerebrospinal fluid enzymes on a more experimental basis.

I. THE ENZYMES OF NORMAL CEREBROSPINAL FLUID

The question of the source of the enzymes of normal cerebrospinal fluid must be answered in terms of the source of the cerebrospinal fluid itself, more particularly in terms of the source of the proteins and cells of the cerebrospinal fluid. The latter, however, is still unsettled. According to Dandy and Blackfan,[2] Weed,[3] and Cushing,[4] the fluid is derived from the choroid plexus within the ventricles, and is absorbed from the subarachnoid space chiefly into the cerebral sinuses. Merritt and Fremont-Smith,[5] as a result of their chemical studies, be-

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lieve with Mestrezat that the cerebrospinal fluid is best understood if it is looked upon as a dialysate of the blood plasma. On the other hand, Hassin[6, 7] maintains, on the basis of histopathologic studies, that the cerebrospinal fluid consists of tissue fluids which pass out from the neural tissue along the perivascular spaces into the subarachnoid space and perhaps the ventricles. At present it is generally considered, according to Flexner,[8] that ". . . the cerebrospinal fluid probably arises in large part from the highly vascular choroid plexuses. To fluid from this source is added a relatively small, perhaps insignificant, amount of fluid from the ependymal cells of the ventricles. The ventricular fluid from these origins slowly flows into the subarachnoid space where it receives an addition, unknown in quantity, but probably small, from the perivascular spaces of the brain, and, in the opinion of some, from the blood vessels traversing the subarachnoid space."

It is impossible to determine from the foregoing data whether the blood plasma or the neural tissue is the more important source of the cerebrospinal fluid enzymes. According to Boyd,[9] the lymphocytes of normal cerebrospinal fluid appear to pass from the perivascular spaces into the subarachnoid space. This, together with the fact that lumbar and cisternal fluids are richer in protein and cells than is ventricular fluid, makes it seem likely that a considerable portion of the enzymes comes from the neural tissues. Whether this portion is greater than that coming from the choroid plexuses and the blood vessels traversing the subarachnoid space seems impossible to settle at present because of our inability to study the enzymes of the neural tissue fluid.

II. ENZYMES OF THE CEREBROSPINAL FLUID IN MENINGITIS

A. *Leucocytic Enzymes.*—It was noticed that the tryptic activity of the cerebrospinal fluid of purulent meningitis was related to the polymorphonuclear leucocyte content of the fluid.[1, 10] Although no exact correlation was possible, fluids with high polymorphonuclear content generally had greater tryptic activity than fluids with low polymorphonuclear content. In order to test the effect of the presence of polymorphonuclear cells, a sample of lumbar fluid from a patient with streptococcic (hemolytic) meningitis was divided into two parts, one of which was filtered through a Seitz filter. The tryptic activity of the original untreated fluid and of the filtrate were then determined.* The results, together with those obtained with a second sample of fluid from the same patient,

TABLE I

PATIENT	PORTION STUDIED	DIAGNOSIS	DESCRIPTION OF FLUID	CELL COUNT	TRYPSIN (UNITS)
1. J. D.	(a) Original fluid	Streptococcic meningitis	Lumbar, turbid	2,290	0.53*
	(b) After Seitz filtration		Clear	0	0.0
2. J. D.	(a) Original fluid	Streptococcic meningitis	Lumbar, opalescent	440	0.20
	(b) After Seitz filtration		Clear	0	0.0

*Values of this order of magnitude represent relatively high tryptic activity.

*The methods used for the determination of enzymatic activity, together with a description of the units employed, are described in detail in reference 1.

are given in Table I. It is seen that removal of the polymorphonuclear cells by Seitz filtration caused the disappearance of tryptic activity.

In order to ensure that the removal of the tryptic activity by Seitz filtration was not due to adsorption of enzyme by the filter pad, we investigated the effect of centrifugation on the tryptic activity of fluids from patients with purulent meningitis. In addition, the sediment resulting from the centrifugation of one fluid was taken up with an amount of physiologic saline such that the volume of the resulting suspension was equal to that of the original fluid sample. The antitryptic power of the various portions was also determined. The results are given in Table II.

TABLE II

PATIENT	PORTION STUDIED	DIAGNOSIS	DESCRIPTION OF FLUID	CELL COUNT (POLYS.)	TRYPSIN (UNITS)	ANTI-TRYPSIN UNITS %
1. A. McQ.	(a) Original fluid	Influenzal meningitis	Lumbar, turbid	7,200	0.65	40
	(b) After centrifugation		Clear		0.0	40
2. A. McQ.	(a) Original fluid	Influenzal meningitis	Cisternal, opalescent	2,000	1.20	8
	(b) After centrifugation		Clear		0.03	8
3. R. W.	(a) Original fluid	Influenzal meningitis	Lumbar, turbid	5,000	1.40	34
	(b) After centrifugation		Clear		0.25	40
	(c) Sediment taken up with NaCl		Turbid		1.20	0

In each case the tryptic activity of the fluid was greatly decreased by centrifugation, while the antitryptic power was unaffected. In the case of R. W. nearly all the tryptic activity was found in the sediment.

Since the results indicated clearly that the source of the trypsin of the cerebrospinal fluid of purulent meningitis is the polymorphonuclear pleocytosis, we thought that extension of the centrifugation studies to other enzymes might yield information about their sources. It was difficult to obtain sufficient fluid at a single puncture for such studies in addition to the routine cellular, chemical, and bacteriologic studies. However, we succeeded in obtaining enough data to indicate approximately the relative importance of the different sources of the enzyme studied. In Table III are given the data for 5 fluids.

The results for trypsin agree with those of the previous experiments and require no further discussion at this point. In 4 out of 5 fluids centrifugation caused a marked decrease in the phosphatase activity, while in one fluid the activities of the original fluid, supernatant fluid after centrifugation, and sediment were practically equal. The reductions in the phosphatase activity of the fluids were relatively less than the corresponding reductions in tryptic activity. Lipolytic activity was reduced by centrifugation. The activity of the supernatant fluid varied from 0 to about 50 per cent of that of the original fluid, while the average sediment activity was about 50 per cent that of the original

fluid. Tributyrinase activity in the fluid was reduced by centrifugation to values from 50 to 93 per cent of those of the original fluid. The sediment had about half as much activity as the original fluid. Results obtained with esterase and amylase were similar to those obtained with tributyrinase, except for some instances where relatively low enzyme values were not affected by centrifugation. The antitryptic power of the fluids studied was not decreased by centrifugation. In fact, in one case the fluid after centrifugation had a very high antitryptic effect (82 units), and a much lower effect (14 units) when cells were present. In no case was any antitrypsin found in the sediment. If there is any antitryptic substance in the cells, its effect is entirely overcome by the polymorphonuclear trypsin.

The effect of centrifugation on the enzymatic activity of the cerebrospinal fluid of purulent meningitis can perhaps be most easily understood in terms of Willstätter's work on the lyo and desmo forms of leucocytic trypsin and amylase. Willstätter[11] has distinguished between two types of leucocytic enzymes according to the ease with which they are extracted from the cellular material. Lyoenzymes, which are easily extracted from the cells, are considered to be freely dissolved in the cells. Desmoenzymes, which are extracted with greater difficulty, are thought to be firmly bound to the cellular protoplasm. The desmoenzymes are almost always bound to cell complexes and are active in this condition. According to Willstätter and Rohdewald,[12] only about 7 to 11 per cent of the trypsin of polymorphonuclear leucocytes occurs as lyotrypsin, the remainder occurs in the desmo form. This would explain why removal of the polymorphonuclear cells by centrifugation removes nearly all of the tryptic activity of the fluid. At most, the fluid after centrifugation had 15 to 17 per cent of the tryptic activity of the original fluid; the average tryptic activity after centrifugation was about 9 per cent of the original activity, agreeing very well with the lyotrypsin values given by Willstätter and Rohdewald.

The work of Willstätter and Rohdewald[11] on leucocytic amylase indicated that this enzyme occurs to a much greater extent in the lyo form than does leucocytic trypsin. Our results with the amylase of meningitis cerebrospinal fluid are in agreement with this finding, since the sediment after centrifugation had relatively less, and the clear liquor more, activity than was the case with trypsin.

At this point an interesting application of our work can be made. The fact that centrifugation of fluid removes a large part of the phosphatase activity indicates that phosphatase is firmly bound in the polymorphonuclear leucocyte, probably more so than amylase, but less firmly than trypsin. Also, our results indicate that the polymorphonuclear cells are very rich in lipase, tributyrinase, and esterase, in agreement with the findings of Fleischmann.[13] Further, the fact that the sediment studied after centrifugation contains these enzymes in considerable quantity indicates that they occur to an important extent in the desmo form. It is difficult, however, to obtain any idea of the relative amounts of lyo and desmo forms of leucocytic lipase, tributyrinase, and esterase, since we have thus far been unable to distinguish between these lyoenzymes and

plasma enzymes which may have entered the cerebrospinal fluid because of increased meningeal permeability.

B. *Effect of Increased Meningeal Permeability: Antitrypsin.*—It is well known that there is an increase in meningeal permeability in meningitis.[14] It is possible, therefore, that some enzymes may enter the cerebrospinal fluid from the blood plasma in meningitis. So far, no direct measure of the enzymatic activity derived from this source is available. However, study of the antitrypsin (or trypsin-inhibitor) of meningitic fluid may throw some light on this subject.

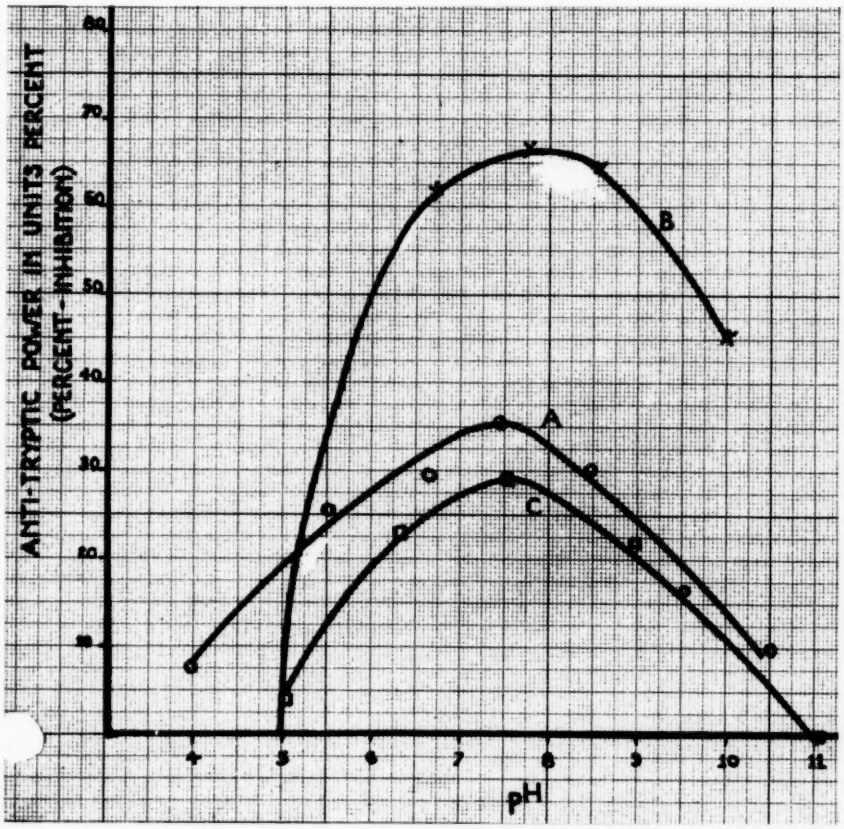

Fig. 1.

Chrometzka[15] has found that the antitryptic power of blood serum varies with pH, having a maximum value of approximately pH 8. On either side of this region the antitryptic power decreases until it disappears at pH values 4 and 12. It is interesting to see whether the antitrypsin found in meningitic cerebrospinal fluid depends in a similar way on pH. If so, it would point to the hematogenous origin of the pathologic antitrypsin, particularly since no antitrypsin is found in normal cerebrospinal fluid.

Accordingly, we studied the effect of change of pH on the antitryptic power of fluid from a patient with hydrocephalus complicated by *Staphylococcus*

TABLE III

CASE	DIAGNOSIS	PORTION STUDIED	APPEARANCE	CELL COUNT	TRYPSIN (mM TYROSINE)	ANTITRYPSIN UNITS	PHOSPHATASE (MG. PHOSPHORUS)	LIPASE (mM FATTY ACID)	TRIBUTYRINASE (mM FATTY ACID)	ESTERASE (mM FATTY ACID)	AMYLASE (mM MALTOSE)
D. R. (1)	Meningococcic meningitis	(a) Lumbar fluid	Turbid	Poly. 3,000	0.67	7	14.4	2.5	2.2	0.6	0
		(b) Supernatant fluid after centrifugation	Clear		0.03	10	5.2	1.3	1.4	0.9	0
D. R. (2)	Meningococcic meningitis	(a) Lumbar fluid	Cloudy	Poly. 1,200	0.40	0	8.0	1.3	3.4	0.9	0.8
		(b) Supernatant fluid after centrifugation	Clear		0	0	1.3	0.7	1.7	0.9	0.8
R. K. (1)	Hydrocephalus B. pyocyaneus pyocephalus	(a) Ventricular fluid	Turbid	Frank pus	2.80	14	33.6	1.3	9.8	4.2	19.1
		(b) Supernatant fluid after centrifugation	Bluish green		0.43	82	9.6	0	9.1	2.9	8.8
		(c) Sediment taken up with NaCl	Turbid		2.33	0	60.2	0.3	4.7	2.5	9.9
R. K. (2)	Hydrocephalus B. pyocyaneus pyocephalus	(a) Ventricular fluid	Turbid	Frank pus	2.32	26	66.4	1.1	7.4	3.9	0
		(b) Supernatant fluid after centrifugation	Bluish green		0.27	0	22.9	0.2	4.9		
		(c) Sediment taken up with NaCl	Turbid					1.0	3.0	1.6	
W. B.	Cerebral abscess	(a) Lumbar fluid	Turbid	Poly. 34,000			55.0	2.4	5.0	3.9	
		(b) Supernatant fluid after centrifugation	Clear				55.4	0.4	3.3	1.7	
		(c) Sediment taken up with NaCl	Turbid				52.4	1.2	3.7	3.5	

aureus pyocephalus. The fluid was divided into several portions to which were added 1 or 2 drops of sodium hydroxide or hydrochloric acid solutions of different strength. After ten or fifteen minutes the antitryptic power of the various portions was determined by the method described in our previous paper. The results are plotted as curve A in Fig. 1. It is seen that the antitryptic power has a maximum of approximately pH 7.5 and falls off on either side of this estimate. At pH values of 4 and 11 the antitryptic power is small but has not completely disappeared. It was thought that this may have been due to the short time (ten to fifteen minutes) allowed for the action of the acid and alkali on the cerebrospinal fluid. The work was repeated on two other fluids, one from the same patient, and the other from a patient with a clinical diagnosis of tuberculous meningitis. In these instances the fluid portions were treated with acid or alkali for an hour. The results are plotted as curves B and C of Fig. 1. It can be seen that the antitryptic power has a maximum value after incubation at pH 7.0 to 8.0 and falls off on either side of this range, vanishing at about pH 4 and pH 11.

It follows from the foregoing results that the antitryptic power of meningitic cerebrospinal fluid depends on pH in the same way as the blood serum antitrypsin. Since the normal cerebrospinal fluid contains no antitryptic substances, the antitrypsin of meningitic fluid must come either from the blood plasma or from the polymorphonuclear leucocytes. However, it has been found (see Tables II and III) that centrifugation of meningitic fluid has no effect on the antitryptic power of the fluid, and that the cellular sediment itself exhibited no such power. It is probable, therefore, that the antitrypsin of meningitic cerebrospinal fluid enters the fluid from the plasma because of the increased meningeal permeability.

C. *Enzymes of the Cerebrospinal Fluid in Tuberculous Meningitis.*—Since the cerebrospinal fluid of tuberculous meningitis differs from that of purulent meningitis in the number of polymorphonuclear cells, it would be expected that this difference might account for the observed difference in enzymatic properties. This is borne out by the fact that the polymorphonuclear cells have been shown to be responsible for the presence of tryptic activity and to a large extent for the presence of phosphatase. The enzymatic activity of the cerebrospinal fluid of tuberculous meningitis, which is not due to the polymorphonuclear cells, is probably due in part to the passage of plasma enzymes across the meningeal barrier, and in part to the presence of lymphocytes which are known to be particularly rich in lipolytic enzymes.[16]

III. ENZYMES OF THE CEREBROSPINAL FLUID IN HYDROCEPHALUS AND BRAIN TUMOR

We have pointed out[1] that our specimens of cerebrospinal fluid in hydrocephalus, brain tumor, brain abscess, and brain cyst can be divided into two groups. The first group contains those cases in which meninges or ventricles are known to have been invaded by a neoplasm, or by hemorrhage, or inflamed. The second group contains cases in which there was no known involvement of meninges or ventricles. Some typical examples of fluids of the two groups are given in Table IV.

TABLE IV

CASE	DIAGNOSIS	APPEARANCE	CELL COUNT	TRYPSIN (mM TYROSINE)	ANTI-TRYPSIN UNITS	PHOSPHATASE (MG. PHOSPHORUS)	LIPASE TRIBUTYRINASE ESTERASE (mM FATTY ACID)			AMYLASE (mM MALTOSE)	TOTAL PROTEIN MG. PER CENT
					First Subgroup						
W. W.	Hydrocephalus medulloblastoma of cerebellum, invasion of fourth ventricle	Xanthochromic, lumbar	Lymph. 40	0	93	5.8	0.5	2.6	0	0.7	613
B. K.	Hydrocephalus, B. pyocyaneus pyocephalus, spina bifida	Frank pus, ventricular		2.32	26	66.4	1.1	7.4	3.9	0	2,500
W. H.	Hydrocephalus, Staph. aureus pyocephalus, spina bifida	Opalescent, ventricular	Poly. 310	0.04	65	6.9	1.0	2.1	0	0	680
					Second Subgroup						
J. M.	Hydrocephalus, glioma of cerebellum, no invasion of ventricles or meninges	Clear, ventricular	Lymph. 3	0	0	0.8	5.4	2.3	0	0	13
(1) M. R.	Hydrocephalus	Clear lumbar	Lymph. 20	0	0	1.0	0.8	3.0	0		16
(2) M. R.	Hydrocephalus	Clear lumbar	Lymph. 1	0	6	1.1	0.4	1.3	0	0	17
B. S.	Hydrocephalus	Clear ventricular		0	0	0.6	3.0	1.4	0	0	41

As the data indicate, fluids of the first group are usually abnormal in appearance, have elevated cellular and protein contents, and have high phosphatase, lipase, tributyrinase, and antitrypsin values. The fluids of the second group are normal, except for high lipase and tributyrinase values. The enzymatic properties of fluids of the first group can, to some extent, be explained by the presence of polymorphonuclear leucocytes, or by invasion of the meninges by a tumor. The lipase and tributyrinase of fluids of the second group cannot be accounted for on these bases since there is no significant pleocytosis and no increased meningeal permeability (i.e., antitrypsin, phosphatase values, total protein are normal). It is necessary, therefore, to seek a different source for these enzymes. In the following discussion we shall suggest the possibility that these enzymes may be associated with degeneration of nervous tissue.

The pathology of the progressive cerebral atrophy secondary to hydrocephalus has been described by Penfield,[17] by Penfield and Elvidge,[18] and by Hassin.[19] The lateral ventricles are greatly distended. There is much nerve tissue destruction, that of the white matter next to the ventricles being more rapid than that of the gray matter. The corpus callosum is raised and thinned, while the septum pellucidum often disappears. In severe hydrocephalus the corpus callosum may be entirely obliterated, the subcortical white matter destroyed, and the optic chiasm, optic tracts, and tuber cinereum extremely flattened.[20-22] Large numbers of gitter cells are found in the corpus callosum, cerebral parenchyma, subependymal areas, and Virchow-Robin spaces. Hassin[22] has studied the histologic changes in the brain secondary to the increased intracranial pressure caused by extracerebral as well as intracerebral tumors. He found diffuse degenerative changes in the corpus callosum, optic nerve, chiasm, and optic tract, that is, the changes were similar to those found in the pressure atrophy of hydrocephalus.

Penfield[23] has studied the formation and activity of gitter cells in areas of cerebral softening surrounding a glioma. These cells contain neutral fats, fatty acids, and other products of the (enzymatic) breakdown of nerve tissue and demyelination. They carry their contents to the adventitial spaces of the blood vessels where they lose their granular and vacuolar appearance, decrease in size, and finally leave the vicinity of the vessels in characteristic form for renewed phagocytosis. Apparently some of the digestive contents of the gitter cells pass into the perivascular spaces from which they are transported in the tissue fluids to the subarachnoid space. It is possible that in this way lyolipolytic enzymes of the gitter cells and myelophages may reach the cerebrospinal fluid.

Another possible explanation of the presence of lipolytic enzymes is in terms of the enzymes of the brain tissue itself. Recent unpublished experiments in our laboratory indicate that fresh minced brain tissue possesses considerable lipolytic activity. It seems possible that the destructive processes may cause the liberation of lipolytic enzymes from the tissue. The enzyme may then reach the cerebrospinal fluid as indicated above. In this connection it is interesting to note that Spiegel-Adolf[25] has recently reported that in certain neurologic conditions apparently normal fluids may show evidences of brain tissue destruction when conductivity and interferometric methods of analysis are used. Some

cases of hydrocephalus and brain tumor in her series have protein values which are within normal limits, but, nevertheless, have abnormal "residual interferometric values," indicating brain tissue destruction. These findings seem to be in agreement with those reported in our study.

If the lipolytic activity of the fluids under discussion is due to brain tissue destruction, it would be reasonable to expect increased lipolytic activity of the cerebrospinal fluid in other degenerative diseases of the cerebrospinal fluid, such as multiple sclerosis, Schilder's disease, and subacute combined degeneration of the cord, which are characterized by extravascular demyelination and glial proliferation.

More complete studies of the lipolytic activity, neutral fat, fatty acid, cholesterol, and cholesterol ester contents of cerebrospinal fluid in these diseases may yield valuable information about the chemistry and pathology of nerve tissue degeneration. The fact that high lipase values have been found[1] in isolated cases of multiple sclerosis, carbon monoxide poisoning, and cerebral arteriosclerosis indicates that such studies may be profitable.

The study of multiple sclerosis by such an approach may be of particular interest in view of Brickner's suggestion[24] that the demyelination in this disease is caused by a lipolytic ferment. It is possible that the lipase and tributyrinase which we have found in the cerebrospinal fluid and which we suggest may be involved in nerve tissue degeneration may be related to Brickner's lipolytic enzyme.

CONCLUSIONS

1. There are two possible sources of the enzymes of normal cerebrospinal fluid: (a) the blood plasma and (b) the neural tissue fluid. It is at present impossible to determine the relative importance of the two sources.

2. Centrifugation studies of the cerebrospinal fluid of purulent meningitis indicate: (a) that the tryptic activity of the fluid is due almost entirely to the desmotrypsin of the polymorphonuclear cells; (b) that the phosphatase activity of the fluid is due chiefly to desmophosphatase of the polymorphonuclear cells; (c) that the lipase, tributyrinase, and esterase activities are due in part (about 50 per cent) to desmolipolytic enzymes of the polymorphonuclear cells; (d) that the amylase activity is due, in a lesser degree, to the desmoamylases of the polymorphonuclear cells.

3. The remainder of the enzymatic activity of the cerebrospinal fluid of purulent meningitis is probably due to: (a) lyoenzymes of the polymorphonuclear cells and (b) plasma enzymes which enter the cerebrospinal fluid because of increased meningeal permeability. It has so far been impossible to determine the relative importance of (a) and (b).

4. The antitryptic power of the cerebrospinal fluid of tuberculous meningitis and pyocephalus varies with pH, having a maximum near pH 7.5 to 8.0, and decreasing on either side of that range. This similarity to the behavior of serum antitrypsin suggests that the cerebrospinal fluid antitrypsin enters the cerebrospinal fluid from the plasma because of increased meningeal permeability.

5. The lipase and tributyrinase of the cerebrospinal fluid of hydrocephalus and brain tumor in cases where there is no known involvement of the meninges

or ventricles may in part be lyoenzymes of the gitter cells and myelophages which take part in the phagocytosis of fatty debris of nerve tissue degeneration. Lipolytic enzymes may also be liberated from brain tissue by destructive processes. The enzymes possibly reach the cerebrospinal fluid via the perivascular spaces.

6. In cases of hydrocephalus and brain tumor where the meninges are involved by tumor or inflammation, cells or increased meningeal permeability may cause an increase in the enzymatic activity of the cerebrospinal fluid.

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ON THE VALUE OF A "REPEAT" INJECTION OF TETANUS TOXOID (SECONDARY STIMULUS) IN ACTIVE IMMUNIZATION AGAINST TETANUS

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IN A PREVIOUS communication[1] we reported the results obtained after active immunization of 34 adults by means of a primary course of two injections of tetanus toxoid, alum-precipitated, refined. We now wish to report our follow-up observations on 20 members of this group who were subjected to a third "repeat" (secondary stimulus) injection of alum toxoid. Their immunologic response to the primary course of immunization is shown in Table I.

Before the third dose of 1.0 c.c. of tetanus toxoid, alum-precipitated, refined, was injected, all patients were bled, and the sera were titrated for tetanus antitoxin. All but two showed less than 0.1 unit per cubic centimeter. Cases 9925 and 9953 had 0.1 unit each. Sixteen subjects received the secondary stimulus 272 days after the second dose of toxoid. Four got the third injection 359 days after the primary course of immunization was completed.

A composite picture of antitoxin production was then obtained through serial titrations (Table II).

Of 8 patients tested on the fourth day after the "repeat" injection, only one showed 0.25 unit. The rest had the same control values of less than 0.10 unit. Of 9 subjects tested on the fifth day, all but one showed 0.1 unit or more per cubic centimeter of blood serum. The exception, Case 9941, showed less than 0.1 unit. However, when retested on the seventh day, this patient had 1.0 unit of antitoxin per cubic centimeter of serum. It is interesting to note the definite increase in antitoxin titer that occurred within 48 hours in Cases 9928, 9932 and 10004. On the sixth day all subjects but one showed good protection; Case 9947, who showed less than 0.10 unit, was not retested again until a month had elapsed when he had 0.25 unit. This subject was the only member of the group who showed a rather poor antitoxin response, since he dropped to less than 0.1 unit when retested 183 days after the third injection of toxoid. On the seventh day 15 subjects were tested. They all showed a marked increase in their antitoxin titer, the average value being well above 2.0 units per cubic centimeter of blood serum. The magnitude of the increase in many cases was at least 50 times the control value. Between the sixth or seventh and the thirtieth day following injection of the secondary stimulus, 11 subjects showed a further increase in titer, 4 had the same antitoxin level, and 1 person showed a decrease in value. Four individuals actually tested 5 units or more per cubic centimeter of blood serum. To obtain this amount of antitoxin in the blood by passive immunization, a hypodermic injection of about 75,000 units of tetanus antitoxin is needed.

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TABLE I

TETANUS TOXOID, ALUM-PRECIPITATED, REFINED No. 5969-1

(Two Doses Given 92 Days Apart)

Titer expressed in units of tetanus antitoxin per c.c. of blood serum

CASE	CONTROL TITER	DAYS AFTER FIRST DOSE TITER	DAYS AFTER SECOND DOSE TITER				
9945†	+0.01	30 days 0.10	7 days -0.10	15 days +0.25	91 days 0.01	181 days 0.003	272 days 0.01
9939†	0.003	30 days -0.003	15 days +0.25	91 days 0.25	181 days +0.01 -0.10	272 days +0.01 -0.10	
9942†	+0.003 -0.01	53 days -0.01	15 days +0.25	91 days 0.25	181 days +0.01 -0.10	272 days +0.01 -0.10	
10002*	0.003	92 days -0.01	15 days 0.10	91 days 0.01	181 days 0.003	272 days +0.01 -0.10	
9928†	+0.003	53 days 0.01	7 days +0.25	91 days +0.25 -0.50	181 days 0.10	272 days +0.01 -0.10	359 days +0.01 -0.10
9932†	+0.003 -0.01	30 days 0.003	7 days +0.25	153 days 0.10	181 days 0.10	272 days +0.01 -0.10	359 days +0.01 -0.10
10004*	0.003	30 days 0.003	15 days +1.0 -5.0	91 days 0.50	181 days +0.10 -0.25	272 days +0.01 -0.10	359 days +0.01 -0.10
9931†	0.003	30 days 0.01	15 days +0.25	91 days +0.25 -0.50	181 days 0.10	272 days +0.01 -0.10	359 days +0.01 -0.10
9943†	0.003	68 days -0.003	15 days +0.25	91 days 0.10	181 days +0.01 -0.10	272 days +0.01 -0.10	
9946†	+0.01	68 days -0.01	15 days +0.25	91 days +0.01 -0.10	181 days +0.01 -0.10	272 days +0.01 -0.10	
9948*	-0.003	30 days -0.003	7 days 0.25	91 days 0.25	181 days +0.01 -0.10	272 days +0.01 -0.10	
9933†	0.003	30 days 0.003	7 days +0.25	91 days 0.25	181 days +0.01 -0.10	272 days +0.01 -0.10	
9941*	0.003	30 days -0.003	7 days +0.25	91 days 0.10	184 days +0.01 -0.10	272 days +0.01 -0.10	
9944*	+0.003	53 days -0.01	7 days -0.10	15 days +0.25	91 days +0.003 -0.01	181 days +0.01 -0.10	272 days 0.01
9935*	+0.003 -0.01	30 days -0.003	15 days +0.10 -0.25	122 days +0.01 -0.10	153 days 0.01	181 days 0.01	272 days 0.01
9955†	-0.003	68 days -0.01	15 days 0.25	91 days +0.25 -0.50	181 days +0.01 -0.10	272 days +0.01 -0.10	
9947*	+0.01	92 days 0.01	15 days +0.10 -0.25	91 days 0.01	181 days 0.003	272 days 0.01	
9954*	0.003	53 days 0.01	15 days 2.0	122 days +0.10 -0.25	181 days +0.01 -0.10	272 days +0.01 -0.10	
9925†	+0.003 -0.01	30 days +0.01 -0.10	7 days 0.10	91 days 0.25	181 days +0.01 -0.10	272 days 0.10	
9953*	0.003	68 days 0.01	15 days +1.0 -5.0	91 days 0.25	181 days +0.01 -0.10	272 days 0.10	

*Each dose consisted of 0.5 c.c. of alum toxoid.

†Each dose consisted of 1.0 c.c. of alum toxoid.

TABLE II

TETANUS TOXOID, ALUM-PRECIPITATED, REFINED NO. 8387-3

(Third "Repeat" Dose of 1.0 c.c. Given 272 Days After Basic Course of Immunization Was Completed)

Titer expressed in units of tetanus antitoxin per c.c. of blood serum

CASE	TITER BEFORE THIRD DOSE	DAYS AFTER THIRD OR "REPEAT" DOSE TITER											
		4 DAYS	5 DAYS	6 DAYS	7 DAYS	30 DAYS	45 DAYS	92 DAYS	183 DAYS	274 DAYS	367 DAYS	464 DAYS	560 DAYS
9945†	0.01	0.01	-	-	+0.25 -0.50	+1.0 -3.0	-	0.50	+0.10 -0.25	0.10	0.10	+0.01 -0.10	-
9939†	+0.01 -0.10	+0.01 -0.10	-	-	+2.0 -3.5	+1.0 -2.0	-	+0.50 -1.0	+0.10 -0.25	+0.10 -0.25	+0.10 -0.25	+0.10 -0.25	0.10
9942†	+0.01 -0.10	+0.01 -0.10	-	-	+1.0 -2.0	-	+2.0 -3.5	2.0	+0.50 -1.0	0.50	+0.25 -0.50	+0.25 -0.50	+0.25 -0.50
10002*	+0.01 -0.10	0.01	-	-	+0.25 -0.50	+0.25 -0.50	-	+0.10 -0.25	0.10	0.10	+0.01 -0.10	+0.01 -0.10	-
9928†‡	+0.01 -0.10	+0.01 -0.10	0.10	-	5.0	+5.0 -8.0	-	3.0	1.5	+1.5 -3.0	+0.50 -1.0	-	+0.50 -1.0
9932†‡	+0.01 -0.10	-0.10	1.0	-	+3.0 -5.0	+3.0 -5.0	-	+0.50 -1.0	0.50	0.50	+0.25 -0.50	-	+0.25 -0.50
10004*‡	+0.01 -0.10	+0.01 -0.10	+0.10 -0.25	-	+1.0 -3.0	5.0	-	+1.0 -3.0	0.50	+0.10 -0.25	+0.50	-	+0.50 -0.75

*Basic course of immunization: 2 doses, 0.5 c.c. each, given 92 days apart.

†Basic course of immunization: 2 doses, 1.0 c.c. each, given 92 days apart.

‡Third dose ("repeat") given 359 days after basic course of immunization was completed.

Alum toxoid No. 5969-1 used.

9931†‡	+0.01 −0.10	0.25	1.0	–	+2.0 −5.0	+3.0 −5.0	–	+1.0 −3.0	1.0	+0.50 −1.0	0.50	–	+0.25 −0.50
9943†	+0.01 −0.10	–	+0.25 −0.50	–	+2.0 −5.0	–	+1.0 −3.0	+0.50 −1.0	+0.25 −0.50	+0.25 −0.50	0.25	0.25	+0.10 −0.25
9946†	+0.01 −0.10	–	0.25	–	+1.0 −2.0	+1.0 −2.0	–	+0.25 −0.50	0.25	+0.10 −0.25	0.10	+0.01 −0.10	–
9948*	+0.01 −0.10	–	+0.25 −0.50	–	+3.0 −5.0	+5.0 −7.0	–	+1.0 −3.0	+0.50 1.0	0.50	+0.25 −0.50	+0.25 −0.50	+0.25 −0.50
9933†	+0.01 −0.10	–	+0.25 −0.50	–	3.0	5.0	–	+1.0 −3.0	+0.25 −0.50	0.50	+0.25 −0.50	+0.25 −0.50	+0.10 −0.25
9941*	+0.01 −0.10	–	+0.01 −0.10	–	1.0	3.0	–	1.0	0.50	0.25	+0.10 −0.25	+0.10 −0.25	+0.10 −0.25
9944*	0.01	–	–	+0.25 −0.50	–	0.50	–	0.25	0.10	0.10	+0.01 −0.10	+0.01 −0.10	–
9935*	0.01	–	–	+0.10 −0.25	–	+0.10 −0.25							
9955†	+0.01 −0.10	–	–	0.25	–	+1.0 −3.0	–	+0.50 −1.0	+0.25 −0.50	0.25	+0.10 −0.25	+0.10 −0.25	0.10
9947*	0.01	–	–	−0.10	–	0.25	–	0.25	+0.01 −0.10	+0.01 −0.10	+0.01 −0.10	+0.01 −0.10	
9954*	+0.01 −0.10	–	–	–	+1.0 −2.0	–	3.0	+1.0 −3.0	+1.0 −2.0	2.0	+0.50 −1.0	+0.50 −1.0	+1.0
9925†	0.10	–	–	+1.0 −3.0	–	+3.0 −5.0	–	+1.0 −2.0	+0.25 −0.50	1.0	+0.25 −0.50	+0.25 −0.50	+0.25 −0.50
9953*	0.10	–	–	–	+1.0 −3.0	–	2.0	1.0	0.50	0.25	0.25	+0.10 −0.25	0.25

When retested 90 days after the "repeat" injection, there was noted a definite decrease in the antitoxin titer in practically all cases, although it was still well above the minimum protective level of 0.1 unit. Thereafter a more gradual loss of antitoxin occurred. At the end of one year only 3 out of 19 subjects showed less than 0.1 unit of antitoxin per cubic centimeter. Three months later two additional subjects dropped to less than 0.1 unit. The latest titration, done 18.5 months after injection of the third dose of alum toxoid, showed that 14 members of this group still retained a protective titer of 0.1 unit or more, with an average of more than 0.25 unit per cubic centimeter.

COMMENT

The injection of a third dose of alum toxoid in subjects who had been previously actively immunized against tetanus is associated with a rise in antitoxin titer of a surprisingly high magnitude. This rise takes about five days to reach the level of 0.1 unit which is present in the blood serum of persons passively immunized with 1,500 units of tetanus antitoxin.[2] It is stated that the period of incubation of tetanus in man is usually from six to fourteen days, and is directly proportional to the amount of toxin and the severity of the disease. However, shorter periods of incubation, six days or less, are known to occur. It is reasonable to expect that the injection of the "repeat" dose or secondary stimulus will prevent these cases of acute tetanus (short incubation), since we are dealing here with a rapidly increasing titer due to active immunity. Also the value of 0.1 unit which we believe necessary for protection against tetanus[2] is rather a conservative one.

A comparison of Tables I and II reveals the interesting fact that in this series of cases the injection of the "repeat" dose was followed by an antitoxin immunity that persisted above the 0.1 unit level for a definitely longer period of time than was the case after the primary course of immunization. Thus, after the latter (Table I) 5 subjects out of 17 dropped to less than 0.1 unit when tested three months after the second dose, 16 subjects out of 20 showed less than 0.1 unit when bled six months after the second injection of toxoid, while 18 members of the group had less than 0.1 unit when tested nine months after completion of the course. On the other hand, after the "repeat" injection of toxoid was administered to this group of patients, only one subject out of 19 dropped to less than 0.1 unit when tested six months later. Previous titrations showed that all subjects enjoyed good protection. This prolonged maintenance of a protective titer is also reflected in subsequent tests. Thus 14 subjects still showed 0.1 unit or more per cubic centimeter of blood serum as late as eighteen months after the injection of the secondary stimulus.

Although the number of subjects studied is small, this finding of a more prolonged antitoxin response following injection of the secondary stimulus is highly significant, and may have important practical implications if confirmed in a large series of cases. In our original study[1] we found that subjects who received 2 doses, 1.0 c.c. each, of alum toxoid seemed to develop a higher and more lasting antitoxin titer than those who received 2 doses of 0.5 c.c. each. It is of interest to note that following the third "repeat" injection of alum

toxoid, the 3 subjects who dropped to less than 0.1 unit before the year was up, were persons who had received 2 doses of 0.5 c.c. for their primary course of injections. In the absence of a skin test to determine the state of antitoxin immunity, the individual variability in antitoxin response makes it necessary to administer 1.0 c.c. of tetanus toxoid, alum-precipitated, whenever an injury occurs after completion of the primary course of immunization. This naturally brings up the question of sensitization. The toxoid does not contain any horse serum, but it does have traces of beef broth and peptone which may give rise to allergic reactions in sensitive subjects. Fortunately, the number of such cases is small. It must be remembered, however, that alum preparations of protein materials have been successfully used in the laboratory to induce anaphylactic shock in experimental animals. Whether this can occur in man is not known. In our experience covering immunization of over 200 people in the last five years, no untoward reaction has been encountered, except an outbreak of urticaria in one patient following the second injection of toxoid.

CONCLUSIONS

1. One cubic centimeter of tetanus toxoid, alum-precipitated, refined, can be safely injected as a secondary stimulus in subjects who had previously undergone primary immunization against tetanus antitoxin by means of two injections of alum toxoid.

2. Five to seven days elapsed before the antitoxin level of the blood serum reached the minimum protective value of 0.1 unit, following injection of this secondary stimulus ("repeat" or third dose).

3. The injection of this secondary stimulus produced in seven to thirty days a very marked increase in the antitoxin content of the blood. The magnitude of this increase was in many cases at least 50 times the control value. Several subjects had 5.0 units or more of tetanus antitoxin per cubic centimeter of blood serum.

4. Following injection of this secondary stimulus, the antitoxin titer appeared to be maintained above the minimum protective level of 0.1 unit for a longer period of time than was the case after the primary course of two toxoid injections.

5. The occurrence of an injury in subjects that have undergone primary immunization against tetanus requires the injection of a secondary or "repeat" stimulus (1.0 c.c. of alum toxoid).

The Mulford Biological Laboratories, Sharp and Dohme, Glenolden, Pa., gave assistance and valuable advice throughout this study. Dr. E. A. Whitney, medical director of the Elwyn Training School, Elwyn, Pa., gave permission to use inmates of this institution as subjects for the investigation.

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THE HISTIDINE CONTENT ("DIAZO VALUE") OF THE BLOOD IN PEPTIC ULCER*

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THE recent introduction of histidine (Larostidine) therapy for peptic ulcer has been followed by both favorable[1] and unfavorable[2] reports in the literature. A satisfactory explanation of the mechanism or mode of action of injected histidine in experimental or human peptic ulcer is still missing. While the amino acid deficiency theory of Weiss and Aron[3] is attractive, direct experimental proof of its correctness is not available as yet. Chemical experimentation along this line has been meager and inconclusive, due, in part, to the absence of a specific and completely satisfactory method for determining the histidine content of the blood.[4-7]

Diazonium salts have been frequently used to determine colorimetrically certain substances, such as phenols, imidazoles, tyrosine, etc., which, as a group, constitute the "diazo value" of the blood.[8] In a recent paper from this laboratory it was shown that ether-soluble phenols constitute but an insignificant portion of this "diazo value."[9] Schwartz, Riegert, and Bricka[10] state that normal human blood contains no phenols and that histidine is the most abundant imidazole. It seems apparent that any appreciable fluctuations in the histidine content of the blood should be reflected in corresponding changes in the "diazo value."

METHOD

With this idea in mind, the experimental methods outlined in the previous paper[9] were applied to a series of fasting bloods obtained from patients with peptic ulcer who had been admitted to the hospital for treatment. Twenty-four patients, most of whom had duodenal ulcer, were subjected to study. Each of these patients had complained of "stomach trouble," indigestion, epigastric distress, hyperacidity, and other typical ulcer symptoms for periods extending from one to fifteen years. Their ages ranged from 22 to 58 years. The clinical diagnosis in each case was confirmed by roentgen examination. The chemical analyses were generally made shortly after admittance to the hospital. With each ulcer blood, a pooled specimen of oxalated, human blood secured from the hospital laboratory was subjected to simultaneous analysis. The filtrates were prepared by laking 1 volume of blood with 2 volumes of water, followed by 1 volume of 10 per cent sodium tungstate and then by 1 volume of 2/3 N sulfuric acid. It is important to add the reagents in this order, since it was observed that the "diazo values" tend to run about 10 per cent higher if the acid is added to the laked blood before the sodium tungstate. Presumably added free amino and other reactive groups are opened up by the strong acid. It was also found that the precipitated blood proteins are removable either by

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TABLE I

HISTIDINE CONTENT ("DIAZO VALUE") OF ULCER AND CONTROL BLOODS

NO.	AGE	SEX	PERIOD OF ULCER SYMPTOMS (YEARS)	NONPROTEIN NITROGEN MG./100 C.C.		AMINO ACID NITROGEN MG./100 C.C.		BLOOD "DIAZO VALUES"						DIAGNOSIS
								KOESSLER-HANKE METHOD AS HISTIDINE MG./100 C.C.		p-NITROANILINE METHOD AS HISTIDINE MG./100 C.C.		MESCHKOWA METHOD AS HISTIDINE MG./100 C.C.		
				ULCER BLOOD	CONTROL BLOOD	ULCER BLOOD	CONTROL BLOOD	ULCER BLOOD	CONTROL BLOOD	ULCER BLOOD	CONTROL BLOOD	ULCER BLOOD	CONTROL BLOOD	
1	25	M	3	39.5	41.7		7.0	1.2	1.2	4.8	5.5	3.0	2.2	Bleeding gastric ulcer
2	58	M	15	50.0	33.0	7.5	6.1	1.9	1.6	5.6	4.5	2.4	2.2	Gastric ulcer with hemorrhage
3		M		22.0				1.5		4.3				Gastric ulcer
4	56	M	3	38.7	36.4	7.2	7.2	1.4	1.4	6.0	6.4	3.0	2.8	Gastric ulcer
5	26	M	2	37.5	39.1	6.4	6.3	1.1	1.1	4.7	4.9	3.0	2.2	Duodenal ulcer
6	24	F	1	31.6	36.4	6.4	7.2	1.4	1.4	5.7	6.4	2.8	2.9	Ovarian cyst, possible duodenal ulcer
7	38	M	2	30.3	36.0	6.9	7.0	1.3	1.4	6.0	6.3	2.4	2.7	Duodenal ulcer with hemorrhage
8	48	M	2	31.3		6.3		1.0		4.3		2.3		Duodenal ulcer with hemorrhage
9	22	M	4	35.3	33.0	7.0	7.1	1.5	1.4	6.2	6.4	2.8	2.9	Duodenal ulcer
10	37	F	1	33.3	35.3	6.7	6.9	1.4	1.4	6.2	6.4	2.6	2.8	Bleeding duodenal ulcer
11	27	M	5			7.4	7.8	2.1	1.7	6.2	5.1	3.4	2.6	Duodenal ulcer
12	33	M	10					1.6	1.7	4.5	6.0		2.3	Pyloric ulcer with partial obstruction
13	31	M	7	38.3	41.7	6.8	5.5	1.8	1.9	4.3	4.7	2.1	2.2	Duodenal ulcer, nonobstructive
14	36	M	4	30.0		5.4	5.9	1.6	1.6	4.5	4.8	2.0	2.3	Chronic spastic colon; gastrointestinal neurosis
15	27	M	5	31.0	28.0	6.4	6.0	1.9	1.6	5.4	4.5	3.0	2.2	Duodenal ulcer
16	38	F	3	34.0	34.1	6.5	5.8	1.2	1.2	4.5	4.7	2.1	2.4	Duodenal ulcer
17	33	M	2	37.5	30.0	7.3	6.8	1.7	1.4	6.0	5.1	3.2	2.4	Duodenal ulcer
18	45	M	2	29.4	31.3	7.0	6.9	1.5	1.4	4.7	4.9	2.2	2.4	Duodenal ulcer
19	50	M	5	30.0	30.0	6.7	6.8	1.5	1.4	5.5	4.9	2.4	2.3	Duodenal ulcer
20	58	M	2	37.5	30.2	5.6	5.5	1.5	1.3	5.7	4.9	2.5	2.5	Nonobstructive duodenal ulcer
21	28	M	2	33.6	30.0	6.5	5.5	1.3	1.3	5.4	4.9	2.4	2.5	Peptic ulcer following posterior gastroenterostomy
22				31.3	32.6	5.1	5.6	1.3	1.5	4.7	4.9	2.2	2.5	Peptic ulcer
23	40	M	5	33.6	35.0	6.3	7.2	1.1	1.2	4.0	4.5	2.3	2.5	Old cicatrized duodenal ulcer with pyloric obstruction
24	22	M	1	33.3		6.3	5.2	1.0	1.1	5.0	4.5	2.4	2.4	Nonobstructive duodenal ulcer
	Averages			34.05	34.11	6.56	6.44	1.45	1.42	5.18	5.24	2.57	2.46	

filtration or by centrifugation without influencing the various values. In addition, determinations of the nonprotein nitrogen and amino acid nitrogen content of the bloods were also made by the usual Folin colorimetric methods. The data are given in Table I.

COMMENT AND SUMMARY

It is obvious that the values given by the various chemical determinations on the pathologic and on mixed control bloods tend to run parallel. Significant differences between the bloods from ulcer patients and the control bloods are consistently absent, and all values are well within normal limits. The amino acid nitrogen values for the ulcer bloods ranged from 5.1 to 7.5, with an average of 6.56, whereas those for the control bloods ranged from 5.2 to 7.8, with an average of 6.44 mg. per 100 c.c.[4, 5, 12] The "diazo values," as determined by the modified Koessler-Hanke method,[9] and calculated and reported as histidine, ranged from 1.0 to 2.1, with an average of 1.45 mg., and 1.1 to 1.9, with an average of 1.42 mg. histidine per 100 c.c. for the ulcer and control bloods, respectively. By the p-nitroaniline method,[11] values for the ulcer bloods ranged from 4.0 to 6.2, with an average of 5.18 mg. histidine, as compared with 4.5 to 6.4, with an average of 5.24 mg. histidine per 100 c.c. for the control bloods. By the modified Meschkowa procedure[9] ulcer bloods ranged from 2.0 to 3.4, with an average of 2.57 mg., as compared with 2.2 to 2.9, with an average of 2.46 mg. histidine per 100 c.c. for the control bloods. These values are similar to those reported for tungstic acid filtrates in the previous study on the phenol and imidazole content of normal human blood.[9]

Examination of the data shows that no distinguishing differences exist between the values given by the various diazotization procedures on the control bloods and on those from patients with peptic ulcer. There seems to be no appreciable difference between the histidine content of the blood of patients with peptic ulcer and the blood of normal individuals. The data do not tend to confirm the theory that a histidine deficiency exists in the blood of patients with peptic ulcer.

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LABORATORY METHODS

A CONTRIBUTION TO THE QUESTION OF THE DETERMINATION OF NICOTINIC ACID IN URINE

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THE papers of Elvehjem, Fouts, Smith, Spies and his collaborators directed attention to the therapeutic significance of nicotinic acid. It has been said that this substance contains the most effective protective factor against pellagra known. It is no longer doubtful that deficiency of this substance may give rise to pictures which represent the individual symptoms of pellagra, and in the dog this deficiency is able to bring about the black tongue disease. The most simple method of recording these stages clinically would be to determine the content of nicotinic acid of the body fluids.

It was, therefore, natural that attempts were made to obtain more exact data on the occurrence of nicotinic acid in the normal and pathologic human organism. The first investigations on the occurrence of nicotinic acid in the urine are connected with the names of Ackermann, Komori, and Sendju. These workers observed that nicotinic acid, after having been fed to the dog, was excreted partly as free nicotinic acid, partly combined with glycine (as nicotinuric acid) and as trigonelline. As to the methods, the papers of Karrer and Keller opened new paths describing a quantitative method of determining nicotinic acid amide in the organism of the animal by means of dinitrochlorobenzene. At the same time, Vilter, Spies, and Mathews reported that they, too, had succeeded in determining nicotinic acid and some of its derivatives in the urine by means of dinitrochlorobenzene. After having carried out exact experiments these authors found that nicotinic acid, nicotine amide, sodium nicotinate, and nicotinic acid diethylamide produce a substance with dinitrochlorobenzene which yields a red color on the addition of alkali. Neither trigonelline nor picolinic acid give this red color reaction. While Karrer and Keller remarked that they obtained deep-colored yellowish-red derivatives after alkalization, Spies and his co-workers stated that they observed, after addition of alkali, a purple-burgundy red color, in proportion to the quantity of derivatives of nicotinic acid present. If the added alkali is too much concentrated, the color changes to yellow. Moreover, Spies and his co-workers pointed out that the red color tints of nicotinic acid and nicotinic amide differ from each other, even if the two substances have undergone the same preliminary treatment. This occurrence made it necessary to have two different standard color curves for quantitative colorimetric measurement. With reference to the urine examination, this fact represents an obstacle,

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since the presence of different shades of red renders an unmistakable result doubtful. It has not yet been discussed as to what the different red color obtained with dinitrochlorobenzene may be due. All determinations carried out hitherto have had the result that the normal human urine contains 20 to 50 mg. of nicotinic acid in the daily amount.

We also tried to attain a method of determination of nicotinic acid in the urine with dinitrochlorobenzene. Short of slight modifications we proceeded along the same lines as described by Spies. After publication of his method, however, we strictly followed his instructions.

The analysis of the normal individual components of the urine likely to produce the red color after addition of dinitrochlorobenzene, led us, after a number of negative results had been obtained, to a positive one. We found that creatinine yielded a red color with dinitrochlorobenzene in proportion to the concentration of creatinine. But also with creatinine the red color does not appear until alkali has been added to the mixture. The more the creatinine solution is diluted, the more the shade changes from purple to orange. With the help of this method it is possible to determine the presence of amounts of creatinine up to 0.5 mg. (We used creatinine hydrochloride produced by Hoffmann La-Roche, Basle.)

We believe that the fact that creatinine yields a color reaction with dinitrochlorobenzene provides a source of error with reference to the determination of nicotinic acid in the urine, which is performed by means of this reagent. Considering that 500 to 1,000 mg. of creatinine are excreted in the urine daily, while only 20 to 50 mg. of nicotinic acid are discharged, and the presence of both substances can proved to the same extent, creatinine is, therefore, present in amounts 20 to 50 times as great as the other substance. In order to obtain definite results for nicotinic acid itself, creatinine must be removed from the urine. Even by using Lloyd's reagent we have so far not succeeded in finding a reliable method.

ALFASIROAD, P.O.B. 1139

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Addendum.—After this report was completed, E. Bandier and J. Hald (Biochem. J. **33**: 264, 1939) and K. Ritsert (Klin. Wchnschr. **18**: 934, 1939) published papers which provided important information on the problem of determining nicotinic acid in biological material (including blood and urine). Bandier and his collaborators devised a method in which they used cyanogen-bromide-metol (p-methylaminophenol sulfate) while Ritsert suggested a modification of the brom-cyananiline method. Ritsert's results greatly differ from those of other authors on quantitative lines. For example, he found in the normal urine an average proportion of 50-300 gamma in 100 c.c. which would amount to 1-6 mg. in the daily output.

AN IMPROVED SUCTION APPARATUS*

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WATER-SUCTION siphonage has been a recognized therapeutic procedure in surgery for many years. Wangensteen[1] used it with a Levin tube for gastrointestinal decompression. It has also been employed for biliary drainage, suprapubic drainage, stomach drainage, and for many other purposes. Meehling[2] in 1917 described the earliest suction apparatus using water siphonage. In some of the suction apparatus the drainage mixed with the water system. This made an unsightly appearance and did not permit accurate measurement of the amount of siphonage returns. This was corrected by creating a separate water system and a flask for siphonage collection. There are numerous suction siphonage machines that have been patented. These are expensive and involve the use of valves that make its use complicated.

At the University Hospital we have devised an apparatus that is very simple in construction, easily transportable, and inexpensive. It does not require much attention while in operation. The necessary parts are as follows:

1. Two 2,000 c.c. flasks, graduated and capable of being suspended, inverted.
2. Three 2-holed rubber stoppers.
3. Glass tubing, rubber tubing, and glass connecting tips.
4. One liter flask to collect siphonage returns.
5. Metal standard, 54 inches high.
6. One Y glass tube.
7. Metal stopcocks (screw type) and right angle clamps to suspend flasks.

The two 2,000 c.c. flasks comprise the water system which is entirely separate from the siphonage returns. The apparatus is constructed as shown in Fig. 1.

The first 2,000 c.c. flask (*A*) is filled with water to a level just below the breather tube and suspended from the top of the standard. The second 2,000 c.c. flask (*B*) is empty and suspended at the bottom of the standard. The 54 inch standard allows adequate height for efficient suction. The liter flask (*C*) is supported in the middle of the standard. Each 2,000 c.c. flask has a 2-holed rubber stopper, in one hole of which there is a long glass breather tube and in the other a shorter glass connecting tip. On the collecting liter flask there is a 2-holed rubber stopper; in one hole there is a glass Y tube, and in the other a small glass connecting tip. The system is so arranged that water will flow from flask *A* at the top of the standard into flask *B* at the bottom of the

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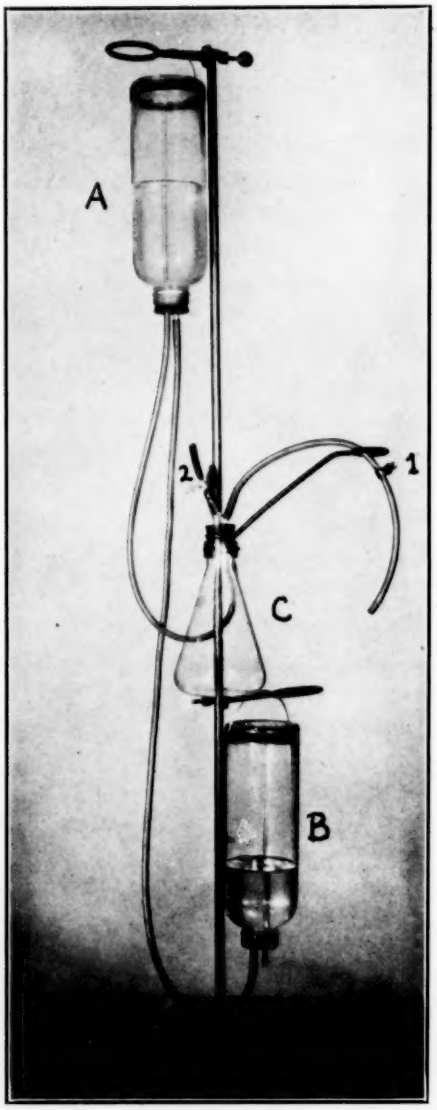

Fig. 1.

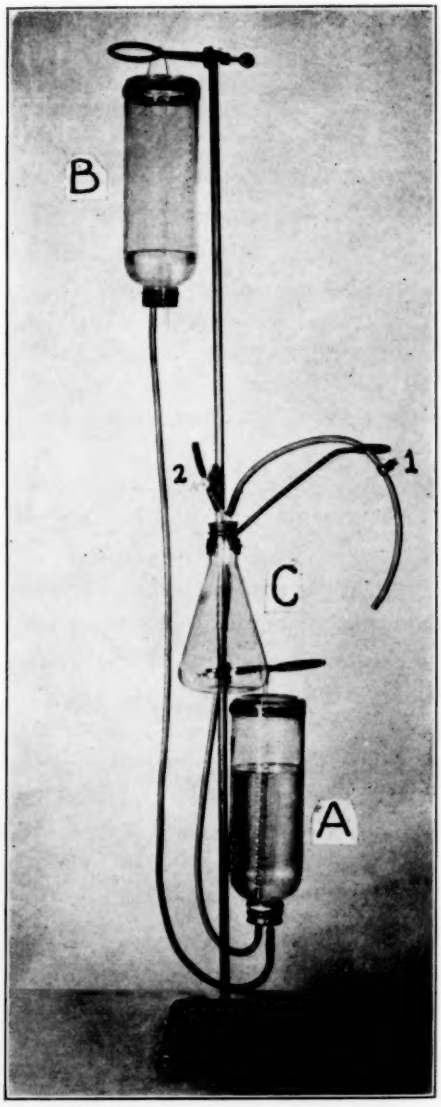

Fig. 2.

Fig. 1.—Apparatus is ready for connection to Levin tube. Flask *A* is at the top of standard, flask *B* is at bottom, and flask *C* is in the middle. Stopcock *2* is on one limb of the Y tube, and stopcock *1* is on tube connecting with Levin tube. Stopcock *2* is closed and stopcock *1* is open. Water is running from flask *A* into flask *B* and creating suction in flask *C*, which is connected to the Levin tube and stomach.

Fig. 2.—Refill the apparatus when flask *A* is empty, then hang flask *A* at bottom of standard and raise flask *B* to the top of standard. Close stopcock *1* and open stopcock *2*. It takes one minute to refill flask *A* without disconnecting the entire apparatus or adding any more water into the system. When flask *A* is refilled, then raise it to top of standard and lower flask *B* to the bottom; close stopcock *2* and open *1*. The apparatus is again ready for use.

standard. As the water flows from flask *A* a vacuum is created above the breather tube in flask *A* that is transmitted to flask *C* which is connected with the Levin tube and stomach. There are two stopcocks in the entire system, one on the tube connecting with the Levin tube (*1*), and the other on one limb of the Y tube. When in operation, stopcock *1* is open and stopcock *2* on Y tube is closed. To refill flask *A*, when the water in flask *A* has emptied into flask *B* one opens stopcock *2* and hangs flask *A* at the bottom of the standard and flask *B* at the top. It takes about one minute to refill the flask without disconnecting any stoppers or adding any more water into the system. When flask *A* is full again, resuspend at the top of the standard, close stopcock *2*, and open stopcock *1*. The system is ready to operate again.

The system will operate for twelve hours and longer without requiring refilling, as each flask holds 2,000 c.c. In refilling it is important to see that stopcock *2* is kept open and stopcock *1* is closed. This prevents air being pumped into the stomach during the water change.

The advantages of this apparatus are as follows:

1. It can be constructed readily and inexpensively by anyone.
2. It does not require much attention from the nurse or person in attendance.
3. The apparatus is complete on one standard and light enough to permit ease in transportation.
4. Accurate measurement of fluid and air siphonage can be made.
5. The water system does not require removal of stoppers and refilling of the flasks.
6. The standard is set on the floor and the drainage returns are out of the patient's sight.

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AN IMPROVED METHOD FOR THE IODIMETRIC DETERMINATION OF PYRUVIC ACID*

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BECAUSE of the important position which pyruvic acid occupies in metabolism, a reliable method for its chemical determination should be available. Acidimetric analyses of biologic mixtures being necessarily impractical, it appears that a method based upon the reactivity of the carbonyl group should offer a better approach. Unfortunately, there is no known compound which is specific in its reaction with the carbonyl group of pyruvic acid. Consequently, if other alpha keto acids, ketones, or aldehydes are present, they will be included in the analysis. Despite this lack of specificity, a measurement of the total carbonyl content of a biologic mixture affords a valuable means of following the course of pyruvate metabolism. If a more positive identification of the carbonyl content is required, it is necessary to resort to characterization studies.

The most widely used procedure for the quantitative determination of pyruvic acid is based on the iodimetric estimation of the bisulfite taken up by the carbonyl group. Such has been the basis of the Clift and Cook method.[1] Tests of this method, using pure pyruvic acid, gave uncertain results because of a fading end point when the excess bisulfite was removed with iodine. Moreover, only about 83 per cent of the expected carbonyl content could be determined, as compared with carboxyl determinations giving 98 per cent. The fading end point suggested that the bisulfite-pyruvate solution was unstable at room temperature (24.5° to 33.0° C.), which was in agreement with the observation of Elliott and co-workers.[2] Cooling the bicarbonate, 0.1 N iodine, and the bisulfite-pyruvate solutions to ice temperature[2] completely stabilized the end point and increased the carbonyl value by about 8 per cent. The carbonyl value, however, was still appreciably lower than the carboxyl value.

It seemed that the effervescence occurring when bicarbonate was added to free the bound bisulfite might cause a loss of some of the liberated bisulfite, even in the cold solution. To test this possibility, an excess of 0.01 N iodine was added to the cold bisulfite-pyruvate solution after the initial end point had been reached but prior to liberation of the bound bisulfite with bicarbonate; the excess iodine was titrated against standard thiosulfate. This change in the procedure raised the carbonyl value to about 99 per cent and thus brought it into good agreement with the carboxyl value. The original method[1] was further improved by a material decrease in the amount of bisulfite, with a consequent decrease in total volume and in the amount of 0.1 N iodine required to combine with the excess bisulfite.

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The method as finally adopted, together with tests of its reliability and its application to certain biologic materials, is given below.

DESCRIPTION OF METHOD

Reagents.—(A) 2.5 per cent sodium bisulfite, prepared daily or at frequent intervals.

(B) 0.01 N potassium iodate. 0.3567 gm. of potassium iodate was dissolved in water and diluted to one liter. This served as the primary standard. The solution is stable for several months, if kept in the dark.

(C) 0.1 N iodine (approx.). 3.57 gm. potassium iodate, 45 gm. potassium iodide, and 5 ml. concentrated sulfuric acid per liter of solution.

(D) 0.01 N iodine, prepared daily or at frequent intervals from (C) by dilution.

(E) 0.1 N sodium thiosulfate (approx.). 25.0 gm. $Na_2S_2O_3.5\ H_2O$ per liter of solution, containing 0.5 per cent amyl alcohol.[3]

(F) 0.01 N sodium thiosulfate, prepared daily or at frequent intervals from (E) by dilution.

(G) 2.5 per cent potassium iodide.

(H) 5.0 per cent acetic acid. 50 ml. glacial acetic acid diluted to one liter.

(I) Starch solution. A 1.0 per cent solution of soluble starch in nearly saturated sodium chloride. Solution of the starch was accomplished by heating to near boiling.

(J) Sodium bicarbonate suspension. An excess of sodium bicarbonate was added to water.

(K) Purified pyruvic acid. Eastman C. P. pyruvic acid was purified by triple vacuum distillation in an all-glass still. The third distillation which had a boiling point of 41.5 to 42.5° C. (not corrected) was collected in a flask immersed in an ice bath. A 10.0 per cent water solution was prepared as a stock solution. It was stored in a dark bottle at 3° C., the bottle being covered with a heavy cloth.

The water used in all analytical work was distilled water which was further purified by redistilling over slightly alkaline potassium permanganate.

Procedure.—Standardization of 0.01 N Sodium Thiosulfate.—A 10.0 ml. aliquot of 0.01 N potassium iodate (B) was measured (Ostwald-Folin pipette) into a 6 inch by 1 inch test tube. Five milliliters of 2.5 per cent potassium iodide (G) and 3 ml. of 5 per cent acetic acid (H) were added, and it was titrated immediately with sodium thiosulfate from a 10 ml. burette until the solution became a faint yellow color. Two drops of starch solution (I) were now added, and the titration continued to a faint-blue end point. The titrations were made in triplicate.

Standardization of 0.01 N Iodine.—This was standardized by titration with the 0.01 N sodium thiosulfate. Triplicate or quadruplicate portions, measured from a 10 ml. burette, were used. The titration was carried to the same faint-blue end point as for the sodium thiosulfate standardization.

TABLE I

THE EFFECT OF COOLING THE REAGENTS AND OF ADDING AN EXCESS OF 0.01 N IODINE UPON THE CARBONYL VALUE OF A PURE PYRUVIC ACID SOLUTION*

CONDITIONS OF TEST	TIME AFTER SOLUTION WAS PREPARED	NUMBER OF ALIQUOTS	PYRUVIC ACID		
			AMOUNT IN ALIQUOT†	AMOUNT RECOVERED	
	Days		*Mg.*	*Mg.*	*Per Cent*
Reagents at room temperature	0	6	2.50	2.07	82.8
	7	6	2.50	2.12	84.8
	14	6	2.50	2.01	80.4
	21	6	2.50	2.06	82.4
	28	6	2.50	2.10	84.0
Reagents at ice temperature	0	6	2.50	2.34	93.6
	7	6	2.50	2.27	90.8
	14	6	2.50	2.19	87.6
	21	6	2.50	2.17	86.8
	28	6	2.50	2.35	94.0
Reagents at ice temperature; excess 0.01 N iodine	0	6	2.50	2.49	99.6
	7	6	2.50	2.50	100.0
	14	6	2.50	2.49	99.6
	21	6	2.50	2.47	98.8
	22	6	2.50	2.48	99.2
	28	6	2.50	2.46	98.4
	86	6	2.50	2.45	98.0
	105	6	2.50	2.37	94.8

*Pyruvic acid made to 0.05 N with hydrochloric acid, prior to carbonyl determination.

†Carboxyl titrations were made on triplicate 0.5 gm. samples of pyruvic acid, using 0.5 N sodium hydroxide with methyl red as the indicator. The carboxyl values at 0, 7, 14, 21, and 28 days were 97.9, 98.4, 98.4, 98.4, and 98.3 per cent, respectively.

TABLE II

EFFECT OF ACIDITY UPON PYRUVATE DETERMINATION*

ACIDITY†	NUMBER OF ALIQUOTS	PYRUVIC ACID			COMMENT
		AMOUNT IN ALIQUOT	AMOUNT RECOVERED		
Norm.		*Mg.*	*Mg.*	*Per Cent*	
0.0057‡	4	2.50	2.45	98.0	No effervescence
0.01	4	2.50	2.51	100.4	No effervescence
0.025	4	2.50	2.48	99.2	Very slight effervescence
0.05	4	2.50	2.47	98.8	Slight effervescence
0.10	4	2.50	2.50	100.0	Some effervescence
0.50	4	2.50	2.50	100.0	Appreciable effervescence; less 0.1 N iodine required
1.00	4	2.50	2.40	96.0	Very appreciable effervescence; much less 0.1 N iodine required

*Excess 0.01 N iodine added before sodium bicarbonate addition. Ice temperature reagents.

†Hydrochloric acid was used.

‡No hydrochloric acid was added.

TABLE III

EFFECT OF THE VOLUME OF SODIUM BISULFITE-PYRUVATE SOLUTION UPON PYRUVATE RECOVERY*

TOTAL VOLUME OF SOLUTION	NUMBER OF ALIQUOTS	PYRUVIC ACID		
		AMOUNT IN ALIQUOT	AMOUNT RECOVERED	
Ml.		*Mg.*	*Mg.*	*Per Cent*
2.0	5	2.50	2.53	101.2
4.0	5	2.50	2.52	100.8
6.0	5	2.50	2.55	102.0
11.0	5	2.50	2.50	100.0
20.0	5	2.50	2.50	100.0

*An acidity of 0.05 N with hydrochloric acid; excess 0.01 N iodine added before sodium bicarbonate addition; ice temperature reagents.

Carbonyl Determination.—1. To each of as many 6 inch by 1 inch test tubes as required, placed in an ice bath, was added 1 ml. of 2.5 per cent sodium bisulfite (A). This is sufficient bisulfite to react with about 20 mg. of pyruvic acid.

2. Five milliliter aliquots of pyruvic acid or unknown, containing from 0.1 to 5.0 mg. of pyruvic acid, were added (Ostwald-Folin pipette). All samples containing an unknown amount of pyruvic acid were analyzed in quadruplicate.

3. The solutions were mixed thoroughly and allowed to stand in an ice bath for fifteen minutes or more.

4. Two drops of starch indicator (I) were then added.

5. Ice temperature 0.1 N iodine was added from a pipette until the excess sodium bisulfite was dissipated; a slight excess of iodine, equivalent to about 1 ml. of 0.01 N sodium thiosulfate, was added. A maximum of 4.3 ml. of 0.1 N iodine will be required. Steps 4 and 5 may be carried out on several samples at one time, so long as the analyses are completed within an hour.

6. By means of the 0.01 N iodine and 0.01 N sodium thiosulfate solutions, contained in 10 ml. burettes, the end point was adjusted to any selected shade of faint-blue color.

7. An amount of 0.01 N iodine equivalent to the pyruvate-bound bisulfite, together with an excess of about 1 ml., was added. If the sample contained an unknown amount of pyruvic acid, one aliquot was analyzed by the Elliott and associates'[2] modification of the Clift and Cook procedure[1] so as to ascertain the approximate volume of 0.01 N iodine required by each of the remaining three aliquots.

8. The ice temperature sodium bicarbonate suspension (J) was thoroughly mixed and 5 ml. was added.

9. The excess 0.01 N iodine was titrated with the 0.01 N sodium thiosulfate, and the end point was adjusted to the same shade of blue color as in step 6. This end point should be permanent for at least thirty seconds.

10. The amount of pyruvic acid was calculated by multiplying the net milliliters of exactly 0.01 N iodine by 0.44.

Blanks on Reagents.—These were made according to the same procedure as for the carbonyl determinations, except that 5.0 ml. of 0.05 N hydrochloric acid replaced the pyruvic acid or unknown in step 2. The average value for 30 determinations was equivalent to 0.098 ml. of 0.01 N iodine (Table V).

TESTS OF THE METHOD

The effect of cooling the reagents and of adding an excess of 0.01 N iodine prior to liberation of the bound bisulfite with bicarbonate is clearly shown by the data in Table I. The analyses were made on the purified acid (K). In addition, the data indicate a very slow deterioration in the pyruvic acid; the carbonyl content had decreased by about 5 per cent at the end of 105 days. Biologic tests also showed a somewhat greater toxicity with decrease in carbonyl content.

Clift and Cook[1] suggested that the reaction between bisulfite and various aldehydes and ketones was more nearly quantitative at relatively low acidities.

Their results indicated that the acidity for pyruvic acid determination should not be greater than 0.1 N. The data in Table II show that the addition of excess 0.01 N iodine prior to bisulfite liberation and the use of cold reagents permitted the quantitative recovery of a constant amount of pyruvic acid over a considerable range of acidity. Even though the acidity was great enough to cause a loss of free bisulfite at 0.5 N acidity, the bound bisulfite was not affected. However, when the solution was adjusted to 1.0 N, there was a slight loss in bound bisulfite as well as an appreciable loss in free bisulfite. The wide range in acidity, 0.01 to 0.5 N, is of value in biologic determinations because it eliminates the necessity for accurately adjusting the acidity of the solutions to be analyzed.

Although a final volume of 6.0 ml. is recommended in the procedure, the data in Table III show that this exact volume is not required, if the amount of pyruvic acid remains constant. No difference was found by varying the volume of the bisulfite-pyruvate solution from 2.0 to 20.0 ml. However, with the use of the suggested apparatus and concentration of reagents, as well as for a possibly better uniformity in results, it would be well to use not over 10.0 ml. aliquots of the unknown.

The data given in the various tables were largely obtained without allowing the solution containing an excess of 0.1 N iodine to stand after its addition. The main purpose for such a suggested addition to the procedure is for convenience only; it is time-saving to add the starch indicator and 0.1 N iodine to several samples at one time and then complete the titration. The data in Table IV show that thirty minutes' standing with excess iodine did not greatly

TABLE IV

THE EFFECT OF EXCESS IODINE UPON THE STABILITY OF BISULFITE-PYRUVATE SOLUTION

MATERIAL	EXCESS 0.1 N IODINE IN TERMS OF 0.01 N $Na_2S_2O_3$	TIME OF STANDING WITH EXCESS IODINE	NUMBER OF ALIQUOTS	PYRUVIC ACID		
				AMOUNT IN ALIQUOT	AMOUNT RECOVERED	
	Ml.	*Hr.*		*Mg.*	*Mg.*	*Per Cent*
Pure pyruvic acid*†	0.10-1.50	0-0	6	2.50	2.48	99.2
	1.15-1.50	0.4-0.8	6	2.50	2.49	99.6
	0.75-1.30	2.0-3.0	6	2.50	2.40	96.0
Rat urines†	1.0-2.0	0-0	7	-	0.72	-
	1.0-2.0	0.4-0.8	7	-	0.71	-
	1.0-2.0	2.0-3.0	7	-	0.67	-
Rat urines†	0.1-1.7	0-0	6	-	1.63	-
	1.3-2.0	0.4-0.8	6	-	1.66	-
	1.9-3.0	2.0-3.0	6	-	1.38	-

*Acidity of 0.05 N with hydrochloric acid.
†Ice-temperature reagents.

affect the results, but that two to three hours' standing may lower the apparent pyruvic acid content. This result indicates that the bisulfite-pyruvate solution may slowly react with the iodine. The same results were obtained from a mixture of rat urines as for pure pyruvic acid.

As a final test of the method, pyruvic acid in amounts of from 0.05 to 15.0 mg. was determined in quadruplicate. Two series of analyses were made; in

one series the regular procedure was followed, whereas in the other series an excess of 0.1 N iodine was added and allowed to stand for about thirty minutes. Inasmuch as there was no essential difference in the results obtained from the two series, the results were averaged and are presented in Table V. Definitely low results were obtained with the smallest (0.05 mg.) and largest (15.0 mg.) amounts of pyruvic acid. Although 97.1 per cent of the pyruvic acid was recovered in the 10.0 mg. level, yet 0.3 mg. was lost, whereas in the 0.05 mg. level only 0.01 mg. was lost. However, with the concentration of reagents and apparatus used, the method appeared to be more accurate for amounts of from 0.1 mg. to 5.0 mg. of pyruvic acid. If smaller or larger quantities are to be determined, the concentration of the reagents and the apparatus should be changed so that quantitative recoveries would be obtained.

TABLE V

THE DETERMINATION OF VARIOUS QUANTITIES OF PYRUVIC ACID*

NUMBER OF ALIQUOTS	NET VOLUME 0.01 N IODINE	PYRUVIC ACID		
		AMOUNT IN ALIQUOT	AMOUNT RECOVERED	
	Ml.	*Mg.*	*Mg.*	*Per Cent*
30	0.098	0.00	–	–
8	0.092	0.05	0.04	80.0
8	0.223	0.10	0.10	100.0
8	0.563	0.25	0.25	100.0
8	1.135	0.50	0.50	100.0
8	2.290	1.0	1.01	101.0
8	3.307	1.5	1.46	97.3
8	5.584	2.5	2.46	98.4
8	11.298	5.0	4.97	99.4
8	22.069	10.0	9.71	97.1
4	31.957	15.0	14.06	93.7

*Average of two series of determinations. An acidity of 0.05 N with hydrochloric acid and 5.0 ml. aliquots of pyruvic acid; ice-temperature reagents; excess 0.01 N iodine.

APPLICATION TO CERTAIN BIOLOGIC MATERIALS

The method has been used in this laboratory in a study of pyruvate metabolism in the intact rat following pyruvate treatment.[4] This work was conducted as individual balance tests covering an eight-hour metabolism period, during which time the animals received 6 doses of sodium pyruvate. The amount of pyruvic acid apparently not retained by the animal was determined by analysis of the urine and of the contents of the intestinal tract.

The urine was collected over a small amount (about 0.5 gm.) of trichloracetic acid. The rat was then killed and the intestinal tract with contents was removed, finely minced, washed into a small flask, and about 1 gm. of trichloracetic acid was added. The samples were allowed to stand overnight in a refrigerator and were then filtered individually into 100 ml. volumetric flasks. After thorough washing, the samples were adjusted to volume. For the determination of the pyruvic acid (total bisulfite-binding substances) content, 5.0 ml. aliquots were used; this volume, therefore, represented one-twentieth of the sample. No adjustment of the acidity of the sample was made.

The data in Table VI give the amounts of apparent pyruvic acid (total bisulfite-binding substances) unabsorbed and excreted by normal young rats

TABLE VI

EFFECT OF EXCESS 0.01 N IODINE UPON THE APPARENT PYRUVIC ACID CONTENT OF RAT URINE AND INTESTINAL CONTENT FOLLOWING PYRUVATE TREATMENT*

BIOLOGIC MATERIAL	SERIES†	NUMBER OF RATS	WEIGHT OF RATS	PYRUVIC ACID GIVEN	APPARENT PYRUVIC ACID PER RAT‡	
					NO EXCESS 0.01 N IODINE	EXCESS 0.01 N IODINE
			Gm.	*Mg.*	*Mg.*	*Mg.*
Urine	A	10	55.4	0.0	1.17	1.63
	B	17	60.0	413.6	20.46	23.22
	C	4	55.6	585.0	58.33	61.48
	D	14	65.9	865.7	98.18	104.46
Intestinal content	A	10	55.4	0.0	1.32	1.96
	B	17	60.0	413.6	1.80	2.81
	C	4	55.6	585.0	7.79	8.69
	D	14	65.9	865.7	53.45	58.61

*Ice-temperature reagents.

†The pyruvic acid was given as the nearly neutralized sodium salt at levels of 0, 60, 90, and 120 mg. in 3.0 ml. per 50 gm. of rat for series A, B, C, and D, respectively. The metabolism period covered 8 hours, during which time each rat received 6 treatments. The rats were killed 1.5 hours after the final treatment.

‡Total bisulfite-binding substances, calculated as pyruvic acid.

following several levels of pyruvic acid treatment. Although it is uncertain that the bisulfite-binding substances found in the urine and intestinal content of the control rats (series A) was pyruvic acid, the increases following pyruvic acid treatment can reasonably be attributed to the administered pyruvate. As with the purified pyruvic acid (Table I), the addition of an excess of 0.01 N iodine prior to liberation of the bound bisulfite increased the amount of apparent pyruvic acid. Furthermore, in similar levels the increase was of the same order of magnitude as for the pure acid.

SUMMARY

1. An improved iodimetric procedure for pyruvic acid determinations in solutions of pure pyruvic acid or in certain biologic materials is described.

2. The essential improvements are (a) the addition of an excess of 0.01 N iodine prior to liberation of pyruvate-bound bisulfite with bicarbonate; (b) a decrease in the amount of bisulfite with a consequent decrease in amount of 0.1 N iodine and in total volume of solution; (c) the use of 0.01 N sodium thiosulfate which permitted a more accurate adjustment of the end point prior to and following the liberation of pyruvate-bound bisulfite.

3. With the apparatus used and concentration of reagents employed, 0.10 to 5.0 mg. of pyruvic acid can be quite accurately determined.

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PROTHROMBIN TIME DETERMINATION*

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THE increasing interest in vitamin K (the coagulation vitamin) warrants the publication at this time of the procedures used in the recent studies on the prothrombin time determination and on vitamin K therapy, as reported by Waddell, Bray, Guerry, and Kelley.[1-3] The method of determining the prothrombin time is essentially that described by Quick,[4] with certain modifications designed to make it more acceptable as a routine procedure in the average clinical laboratory. Especially useful is the modification suggested by one of us (Bray[3]) using capillary blood in the study of prothrombin levels in the newly born infant.

PREPARATION OF THROMBOPLASTIN

The brain is removed from a rabbit recently killed by etherizing, or by bleeding, or by injecting air into the veins; brains of rabbits killed by a blow on the head are not satisfactory. The meninges and the blood vessels are carefully removed. The brain is then macerated by means of a spatula and spread in a thin layer over the surface of a glass plate. The macerated brain is dried in the incubator at 37° C., for two to three days.

The dried brain is removed from the glass by scraping with a razor blade and then finely chopped with the blade. This gives a fine flaky material, which is more satisfactory than the compact powder obtained when the dried brain is ground in a mortar. This material keeps well in the dry state at room temperature. Some batches have kept their potency for over two months. The acetone method described by Quick[4] also has been found satisfactory.

Titration of the Thromboplastin.—A 1:20 suspension of thromboplastin is made in physiologic salt solution (0.9 per cent); 0.2 Gm. of the dried brain in 3.8 c.c. of the salt solution is ample for the titration. The suspension is heated at 56° C. for ten minutes, with occasional stirring. The material is then centrifugalized at low speed for a few minutes to throw down the coarse particles. A turbid supernatant fluid is obtained, containing fine particles in suspension. The following dilutions of thromboplastin are set up in 0.5 c.c. amounts: 1:20, 1:30, 1:40, 1:60, 1:80, 1:120, and 1:160. This may be conveniently done in two stages:

(1) Four tubes are set up in a rack, and into the first and second are placed 0.5 c.c. of the 1:20 dilution (i.e., 0.5 c.c. of the supernatant fluid). Into

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tubes 2, 3, and 4 are placed 0.5 c.c. of 0.9 per cent salt solution. From tube 2 after mixing, 0.5 c.c. is transferred to tube 3, and so on, 0.5 c.c. being discarded from the last tube. These dilutions are 1:20, 1:40, 1:80, and 1:160.

(2) Three other tubes are set up. Into each tube is placed 0.5 c.c. of 0.9 per cent salt solution. Into tube 1 is placed 1.0 c.c. of the 1:20 dilution, and after mixing, 0.5 c.c. is discarded and 0.5 c.c. is transferred to tube 2; after mixing, 0.5 c.c. is transferred to tube 3; and after mixing, 0.5 c.c. is discarded. These dilutions are 1:30, 1:60, and 1:120. The dilutions are then arranged in ascending order.

Two or more samples of blood with known prothrombin time, or from normal healthy adults are used for the titration. The titrations are run in duplicate as outlined under "Technique of Prothrombin Time Determination." The titration usually shows an optimum zone of low findings. For routine tests we choose the dilution falling in the middle of this zone, thus giving a safe margin.

Example:

Tube	1	2	3	4	5	6	7
Dilution	1:20	1:30	1:40	1:60	1:80	1:120	1:160
Time in Seconds	18	18	18	18	21	22	24

Dilution used, 1:30. The thromboplastin suspension made up according to the results of the titration may be stored in the icebox and is suitable for use for several days.

PREPARATION OF BLOOD

Either venous or capillary blood may be used. For venous blood 100 × 12 mm. tubes are used. The tubes are calibrated to 2.0 c.c., and 0.2 c.c. of M/10 sodium oxalate solution (1.34 Gm. per 100 c.c.) is placed in the tube which is stoppered with a cork. Several of these may be prepared and kept on hand.

For capillary blood 75 × 10 mm. tubes are used. They are calibrated at 0.5 c.c., and 0.05 c.c. of M/10 sodium oxalate solution is placed in each tube. These may be prepared and stoppered.

In taking venous blood about 3 c.c. of blood are drawn into the syringe, and immediately enough is added to the oxalate tube described above to fill it to the 2.0 c.c. calibration mark. The oxalate and blood are mixed by tilting the tube five or six times. In case there is any difficulty in making the puncture a fresh needle and syringe should be obtained and the puncture made at a new site. This oxalated blood may be placed in the icebox for as long as overnight without making any significant change in the prothrombin time.

When blood is desired from an infant, it may be obtained by puncturing the superior longitudinal sinus through the anterior fontanel, obtaining slightly over 2 c.c. of blood. However, our comparative studies have shown that blood taken from a puncture of the heel gives practically the same results as blood taken from the longitudinal sinus. The preferred site of puncture is the edge of the plantar surface of the heel rather than the fat pad in the center of the heel. The site is cleaned with alcohol and the area coated with

vaseline. After making a satisfactory puncture, blood is obtained up to the 0.5 c.c. mark in the tube described above. This should be done with frequent shaking to keep the blood well mixed with the oxalate. Paraffined tubes may be used, but we have found this precaution unnecessary.

When blood is desired from the umbilical cord, it is usually collected from the cut surface, using the tubes calibrated at 2.0 c.c.

The plasma is obtained after centrifugalizing the blood for five minutes at a relatively low speed.

TECHNIQUE OF THE PROTHROMBIN TIME DETERMINATION

Thin-walled test tubes, 100 × 12 mm., are used. The tubes are conveniently held in a Wassermann rack. By means of the Folin microsugar pipette 0.1 c.c. amounts of well-mixed thromboplastin suspension are delivered to as many tubes as are needed. With a similar pipette 0.1 c.c. of the plasma is added to the thromboplastin suspension. Each test is done in duplicate. This thromboplastin-plasma mixture may be held at room temperature for as long as an hour, without significant change, but should not be kept in the water bath longer than is necessary for carrying out a small number of tests.

A sufficient amount of M/40 calcium chloride solution (0.365 Gm. calcium chloride crystals per 100 c.c. water) contained in a test tube is placed in a 37° C. water bath. When the thromboplastin-plasma mixture is prepared, it is warmed to 37° C. in the water bath and 0.1 c.c. of the warm calcium chloride solution is added by means of a Folin pipette, at the same instant starting a stop watch. The tube containing the mixture should be kept submerged during this procedure. At the end of fifteen seconds and at every two-second interval after that the tube is momentarily taken from the water bath to be observed for clot formation. This is best seen by tilting the tube, as the clot forms in a rounded mass and slides along the side of the tube. The watch is stopped at the first sign of clot formation.

In some markedly dehydrated newborn infants it may not be possible to obtain sufficient plasma from the 0.5 c.c. blood specimen to run the routine determination. In such cases the whole blood may be used for the test. The same technique is used as for the plasma.

In order to check the system a control determination should be made at the beginning of each set of tests. Either fresh blood from a healthy adult or blood with a known short prothrombin time, which has been kept for not longer than twenty-four hours in an icebox, may be used for this control test.

FINDINGS AND DISCUSSION

Our results in the first 100 prothrombin time determinations on normal adults are tabulated in Table I.

The wide variation and high average of the prothrombin time determinations made during the month of February, as compared with the findings in the four following months, suggest that there may be a seasonal variation in the normal level. This study is not extensive enough to make any definite statements, but this point seems worthy of further investigation.

TABLE I

PROTHROMBIN TIME OF NORMAL ADULTS

MONTH	CASES	LOW	HIGH (TIME IN SECONDS)	AVERAGE
Feb.	28	15	40	28.67
March	21	17	25	20.57
April	10	18	25	21.1
May	31	17	27	21.8
June	10	19	24	22.1
Total	100	15	40	23.4

The average prothrombin time, that is, 23 seconds, is the same as that reported by Quick and co-workers,[4] and also by Butt, Snell, and Osterberg.[5]

As reported by Bray and Kelley,[3] the prothrombin time of cord blood in case of normal infants is usually within the range of normal adults. A prolonged prothrombin time of cord blood may occur in hemorrhagic disease of the newly born infant.

The prothrombin time of the normal newly born infant is very variable. It tends to be low and within normal adult range on the first day, rises to a peak higher than the normal adult range between the second and fifth days, and then comes down within the normal adult range after the fifth day. In hemorrhagic disease of the newborn the prothrombin time may be very high; it may also be high without any evidence of bleeding.

When whole blood is used, the results in normal prothrombin times are practically the same as when plasma is used. In cases of prolonged prothrombin time the whole blood may give a longer time than would result from the use of plasma. However, we do not feel that this is objectionable; in fact the dilution of the prothrombin makes for a more delicate test.

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HEMOLYTIC EFFECTS OF ETHYL AND CAPRYLIC ALCOHOL*

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CAPRYLIC alcohol is commonly used to prevent foaming, particularly of blood and other body fluids.[1] Experience has shown that the addition to blood of this substance, even in small quantities, frequently causes hemolysis. This study was designed primarily to determine the hemolytic power of caprylic alcohol. Comparisons were made with water and with ethyl alcohol, both of which are well known hemolytic agents.[2, 3]

PROCEDURE

Ten samples of fresh, defibrinated ox blood and five specimens of hog's blood were used. The hemolytic agents were added directly to the blood in the following proportions (reagent: blood): for water, 1:10, 1:8, 1:6, 1:4, 1:2, 1:1, 2:1, 3:1; for ethyl alcohol, 1:25, 1:10, 1:6, 1:5, 1:4, 1:3, 1:2, 1:1; for caprylic alcohol, 1:1,000, 1:600, 1:500, 1:400, 1:300, 1:200, 1:100, 1:50, 1:25, 1:10. Mixtures showing perceptible darkening, extreme darkening, transparency, and (for the alcohol-blood mixtures) hemolysis in 0.9 per cent sodium chloride solution were noted at intervals of five, fifteen, thirty, forty-five, and sixty minutes. Thorough mixing by gently inverting the tubes preceded each observation.

The various criteria of hemolysis were judged in the following manner. *Perceptible darkening* was determined by comparison with a tube of untreated blood from the same general sample. *Extreme darkening* indicated the plum color characteristic of laked blood. *Transparency* denoted ability to see print clearly through the blood film left by inversion of the test tube. *Hemolysis in 0.9 per cent sodium chloride solution* was tested by transferring a small amount of each blood mixture to a tube containing 5 c.c. of the salt solution.

Resistance to hypotonic laking of red cells from mixtures not completely hemolyzed after one hour was tested by transferring small amounts to sodium chloride solutions having concentrations of 0.42 to 0.90 per cent.

All observations were made at room temperature. The average was 23.9° C., with extremes at 21.5° and 28.0°; comparisons were made at equal temperatures.

RESULTS

Results are summarized in the accompanying tables. Table I shows the dilution of hemolytic agent in blood at which the various criteria of hemolysis appeared in one hour, together with the extremes encountered and the percentage of samples having the median value. Considerable variation occurred with regard to perceptible darkening, which represents the earliest detectable

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stage of hemolysis. Extreme darkening and transparency, indicating virtually complete hemolysis, gave more uniform results. According to the latter criteria, ethyl alcohol showed approximately four times the laking power of water, while caprylic alcohol was fifty times as powerful as ethyl alcohol and two hundred times as powerful as water. Hemolysis in 0.9 per cent sodium chloride solution was recorded only for mixtures containing the alcohols. Results were practically the same as those for transparency. Some slight differences between ox blood and hog's blood are shown in Table I.

TABLE I

DILUTIONS OF WATER, ETHYL ALCOHOL, AND CAPRYLIC ALCOHOL AT WHICH VARIOUS CRITERIA OF HEMOLYSIS APPEAR

CRITERIA OF HEMOLYSIS	WATER			ETHYL ALCOHOL			CAPRYLIC ALCOHOL		
	MEDIAN		EXTREMES	MEDIAN		EXTREMES	MEDIAN		EXTREMES
	Dil.	*Per Cent*	*Dil.*	*Dil.*	*Per Cent*	*Dil.*	*Dil.*	*Per Cent*	*Dil.*
Ox blood									
Perceptible darkening	6	80	4,6	10	100		400	20	200,600
Extreme darkening	1	90	1,2	4	80	4,5	200	80	100,300
Transparency	1	80	1,2	4	100		200	50	100,200
Hemolysis in 0.9% NaCl				4	100		200	70	200,300
Hog's blood									
Perceptible darkening	2	80	2,4	6	100		300	60	300,400
Extreme darkening	1	80	1,2	5	60	4,5	200	100	
Transparency	1	100		4	80	4,5	200	100	
Hemolysis in 0.9% NaCl				4	100		200	100	

Notes: Figures in "dilution" columns represent parts of blood per one part of reagent in the test mixture. Figures in "per cent" columns refer to the percentage of samples having the median value.

While caprylic alcohol was a much more powerful hemolytic agent than ethyl alcohol or water, it was slower in developing its maximal effect. The maximum hemolytic effect of water was reached in five minutes and that of ethyl alcohol in less than fifteen minutes. Caprylic alcohol, on the other hand, caused laking in progressively weaker dilutions during one hour and may not have reached its end point in that time.

The osmotic resistance of cells from mixtures not completely hemolyzed after one hour is shown in Table II, which shows also the direction of change from the control value for individual samples. The recorded figure for osmotic resistance represents in each case the midpoint of the zone between complete hemolysis and imperceptible hemolysis. As only large differences were of interest, no attempt was made to measure fragility of red blood cells with great accuracy. None of the water-blood mixtures showed important alterations in osmotic resistance. In every instance, mixture of ethyl alcohol with blood in proportions of 1:5 caused a large decrease in resistance to hypotonic laking, while 1:10 dilutions resulted in a definite decrease in but one sample out of thirteen. In the case of caprylic alcohol, the great majority of 1:200 mixtures were completely laked, while 1:300 and 1:400 dilutions uniformly exhibited large decreases in osmotic resistance. Smaller but still definite decreases were ob-

served in a majority of 1:500 dilutions with ox blood, and a few even in dilutions of 1:600. Again differences between ox and hog's blood were noted but were considered of doubtful significance.

TABLE II

OSMOTIC RESISTANCE OF RED BLOOD CELLS EXPOSED TO VARIOUS HEMOLYTIC AGENTS FOR ONE HOUR

HEMOLYTIC AGENT	DILUTION	OSMOTIC RESISTANCE			CHANGE IN OSMOTIC RESISTANCE					
		EXTREMES	AVG.	MED.	DECREASE		INCREASE		NO CHANGE	
		Per Cent NaCl	*Per Cent*	*Per Cent*	*Number*	*Per Cent*	*Number*	*Per Cent*	*Number*	*Per Cent*
Ox blood										
Control		0.43-0.52	0.48	0.49						
Water	1	0.43-0.45	0.44	0.44	0	0.0	0	0.0	2	100.0
	2	0.47-0.52	0.49	0.49	0	0.0	0	0.0	8	100.0
Ethyl alcohol	5	0.53-0.85	0.60	0.65	10	100.0	0	0.0	0	0.0
	10	0.46-0.54	0.50	0.50	0	0.0	0	0.0	9	100.0
Caprylic alcohol	300	0.55-0.85	0.72	0.75	7	100.0	0	0.0	0	0.0
	400	0.51-0.85	0.65	0.65	9	100.0	0	0.0	0	0.0
	500	0.47-0.65	0.59	0.60	7	87.5	0	0.0	1	12.5
	600	0.43-0.65	0.53	0.53	3	50.0	0	0.0	3	50.0
Hog's blood										
Control		0.46-0.49	0.47	0.48						
Water	2	0.46-0.49	0.47	0.48	0	0.0	0	0.0	5	100.0
Ethyl alcohol	5	0.65-0.85	0.80	0.76	5	100.0	0	0.0	0	0.0
	6	0.50-0.60	0.58	0.55	3	60.0	0	0.0	2	40.0
	10	0.44-0.58	0.47	0.49	1	25.0	0	0.0	3	75.0
Caprylic alcohol	300	0.80-0.90	0.85	0.85	5	100.0	0	0.0	0	0.0
	400	0.51-0.60	0.57	0.57	5	100.0	0	0.0	0	0.0
	500	0.46-0.52	0.49	0.49	0	0.0	0	0.0	5	100.0
	600	0.47-0.52	0.48	0.49	0	0.0	0	0.0	5	100.0

Notes: Figures in "dilution" column represent parts of blood per one part of reagent in the test mixture. Only samples showing alteration in osmotic resistance in excess of 0.04 per cent sodium chloride are considered as changed in this table.

The results indicate also the proportions of these reagents which may be added to blood without gross hemolysis in one hour. Water failed to produce perceptible darkening in dilutions of 1:8, while 1:6 dilutions showed this in 80 per cent of the ox blood samples. Extreme darkening and transparency never appeared in dilutions of 1:4 but were almost the rule in 1:1 mixtures. Ethyl alcohol uniformly caused some hemolysis in 1:10 mixtures with ox blood but never in 1:25 dilutions. Extreme darkening and transparency failed to appear at 1:6 but were rather frequent at 1:5. With caprylic alcohol, perceptible darkening was never observed in dilutions of 1:1,000 and appeared only once at 1:600. It occurred at 1:500 in 30 per cent, at 1:400 in 20 per cent, and at 1:300 in another 30 per cent of the ox blood mixtures. Extreme darkening never appeared at 1:400 and only once at 1:300. Eighty per cent of the ox blood and all of the hog's blood samples showed extreme darkening at 1:200. Transparency did not develop in dilutions of 1:300 but was present in half of the ox blood and all of the hog's blood samples at 1:200.

SUMMARY

1. The relative hemolytic powers of water, ethyl alcohol, and caprylic alcohol were tested at room temperature over a period of one hour by mixing the hemolytic agents with defibrinated blood in various proportions.

2. Water caused hemolysis rather uniformly in dilutions of 1:1. Ethyl alcohol was found to have approximately four times the laking power of water. Caprylic alcohol had about fifty times the laking power of ethyl alcohol and two hundred times that of water.

3. Resistance to hypotonic laking of cells from mixtures not completely hemolyzed in one hour was unchanged when water was the hemolytic agent, and decreased over a limited range in the case of ethyl alcohol-blood and caprylic alcohol-blood mixtures.

4. The following dilutions of reagent in blood seemed practically devoid of gross hemolytic effects in one hour: for water, 1:8; for ethyl alcohol, 1:25; for caprylic alcohol, 1:600.

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THE LANGE TEST*

II. The Influence of Particle Size and Hydrogen-Ion Concentration of Gold Sols Upon Lange Test Readings on Syphilitic and Tabetic Spinal Fluids

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EXPERIMENTAL results summarized in a previous paper[1] indicate that the sensitivity of gold sols in the Lange test for paresis increases with increase in particle size and decreases with increase in pH. The present paper summarizes the results of a similar study with syphilitic and tabetic spinal fluids. The gold sols used were prepared by the nuclear formaldehyde method in exactly the same manner as in the study with paretic fluids, and all techniques were carried out as previously described.

Table I

Influence of Particle Size on Syphilitic Readings

Syphilitic fluid "B"	pH of sols 8.3
Average particle size in millimicrons	Test readings
43	3344443321
40	2233321000
38	1223310000
36	1223211000
23.5	1223100000

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The results obtained are summarized in Tables I to XV. The eleven sets of syphilitic data were selected at random from a large amount of similar data. The four sets of tabetic data represent all the information obtained on tabetic fluids.

TABLE II

INFLUENCE OF PARTICLE SIZE ON SYPHILITIC READINGS

SYPHILITIC FLUID "C"	pH OF SOLS 6.99
Average particle size in millimicrons	Test readings
43	3332100000
41	1233100000
39.5	1121100000
38	1110000000

TABLE III

INFLUENCE OF PARTICLE SIZE ON SYPHILITIC READINGS

SYPHILITIC FLUID "F"	pH OF SOLS 6.6
Average particle size in millimicrons	Test readings
43	4332100000
41	2233210000
39.5	1122100000
38	1221000000

TABLE IV

INFLUENCE OF PARTICLE SIZE ON SYPHILITIC READINGS

SYPHILITIC FLUID "D"	pH OF SOLS 6.0
Average particle size in millimicrons	Test readings
45	3333210000
43.5	2333210000
38	2233100000
35	1222100000

TABLE V

INFLUENCE OF PARTICLE SIZE ON SYPHILITIC READINGS

SYPHILITIC FLUID "G"	pH OF SOLS 7.4
Average particle size in millimicrons	Test readings
43	2333100000
41	1221000000
39.5	1100000000
38	0000000000

TABLE VI

INFLUENCE OF pH ON SYPHILITIC READINGS

SYPHILITIC FLUID "H"	PARTICLE SIZE 43 MILLIMICRONS
pH	Test readings
7.38	2333100000
7.01	3333100000
6.62	4332100000
6.10	4433210000

TABLE VII

INFLUENCE OF pH ON SYPHILITIC READINGS

SYPHILITIC FLUID "J"	PARTICLE SIZE 41 MILLIMICRONS
pH	Test readings
7.57	1111000000
7.22	1123100000
6.97	1233100000
6.65	2233210000

TABLE VIII

INFLUENCE OF pH ON SYPHILITIC READINGS

SYPHILITIC FLUID "K"	PARTICLE SIZE 41 MILLIMICRONS
pH	Test readings
7.57	1232100000
7.22	1333100000
6.97	2332200000
6.65	2333210000
6.4	3333220000

TABLE IX

INFLUENCE OF pH ON SYPHILITIC READINGS

SYPHILITIC FLUID "L"	PARTICLE SIZE 39.5 MILLIMICRONS
pH	Test readings
7.46	1100000000
6.94	1121100000
6.61	1122100000
6.41	2232100000
6.22	2233210000

TABLE X

INFLUENCE OF pH ON SYPHILITIC READINGS

SYPHILITIC FLUID "N"	PARTICLE SIZE 38 MILLIMICRONS
pH	Test readings
6.99	1110000000
6.50	1122100000
6.18	2232100000
5.60	2233110000

TABLE XI

INFLUENCE OF pH ON SYPHILITIC READINGS

SYPHILITIC FLUID "M"	PARTICLE SIZE 38 MILLIMICRONS
pH	Test readings
8.3	1122210000
8.0	1234311000
7.4	2244321000
6.6	2344431100
6.2	2454442100

TABLE XII

INFLUENCE OF PARTICLE SIZE ON TABETIC READINGS

TABETIC FLUID "A"	pH OF SOLS 7.90
Average particle size in millimicrons	Test readings
42	3444432100
41	2344421000
36	2334421000
30	1233410000
23.5	1122210000

TABLE XIII

INFLUENCE OF PARTICLE SIZE ON TABETIC READINGS

TABETIC FLUID "B"	pH OF SOLS 7.30
Average particle size in millimicrons	Test readings
42	5555543210
41	5555543100
36	3444310000
30	2234310000
23.5	1233310000

TABLE XIV

INFLUENCE OF pH ON TABETIC READINGS

TABETIC FLUID "A"	PARTICLE SIZE 41 MILLIMICRONS
pH	Test readings
7.90	2344421000
7.50	3444431000
7.28	3455432100
6.60	5555541100
6.00	5555554211

TABLE XV

INFLUENCE OF pH ON TABETIC READINGS

TABETIC FLUID "B"	PARTICLE SIZE 41 MILLIMICRONS
pH	Test readings
8.20	2343220000
7.80	3444331000
7.50	4455543210
7.30	5555543100
6.60	5555553110
6.00	5555555422

SUMMARY

The results indicate that, as in the case of paresis so in the case of syphilis, the sensitivity of gold sols in the Lange spinal fluid test increases with increase in particle size and decreases with increase in pH. Although only a limited amount of data is available on tabetic fluids, the results obtained indicate that these fluids respond to changes in pH and particle size of gold sols as do paretic and syphilitic fluids.

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FUNGICIDAL TESTING: A COMPARISON OF METHODS*

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IN A RECENT issue of Drug Trade News,[1] considerable discussion was given to three methods for making fungicidal tests: those advocated by Reddish,[2] by Klarmann,[3] and by Taft.[4] It is desired to comment herein upon these tests and to suggest a method which shows more clearly the power of a vigorous fungus to survive adverse conditions.

In an earlier paper[5] a method which has proved useful in practical testing of fungicidal agents offered for clinical purposes was outlined briefly. This basic method has now been followed for years in our laboratories and has, naturally, been subject to slight modifications in response to continued experience. It seems advisable to report such minor changes and to review the evidence favorable for this method.

In any such test there are two outstanding factors: the fungus and the test substance. Other factors are variable according to conditions; but these two must be definite, repeatable, and reasonably standardized, if results obtained in different laboratories are to be fairly evaluated.

One major point to keep in mind is that fungi of mold type are not closely comparable to bacteria and cannot be handled successfully by the same methods. A mold consists of threadlike parts (hyphae), more or less intricately woven together into a mat of mycelium; and of fruiting portions with a great variety of spores which are comparable to the seeds of higher plants. Some of the hyphae are feeders which by their rootlike parts (rhizoids) can penetrate the substratum and absorb food for the whole plant. Other hyphae (sporophores) grow upright to bear specialized fruiting branches upon which the spores are developed. In fact, a mold more nearly resembles a shrub or tree than it does a bacterium, so far as *structure* is concerned.

A single species of fungus may reproduce by three or four different types of spores, as well as by "slips" or "cuttings" of the mycelium. Any properly made suspension of mold growth will contain not only several types of spores characteristic of the species but also fragments of hyphae, any intact cell of which may form a new plant. Generally speaking, spores are more resistant to fungicidal agents than are the hyphal cells. Clearly, then, a fungous culture which is to be used in a test must be *allowed to grow* under *favorable conditions* for a *sufficient time* to permit it to develop a *typical mature structure*.

Like the higher plants, fungi vary in the length of time required to mature, i.e., to "ripen" their spores. For Monilia and related types, forty-eight to seventy-two hours is sufficient. For many species of Aspergillus, Penicillium,

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etc., seventy-two to ninety-six hours will suffice; but those species which produce *ascospores* should grow ten days to two weeks before being used in suspensions. Among the true dermatophytes—species of Trichophyton, Epidermophyton, Microsporum, Achorion—two weeks is the shortest, four weeks the longest, period of growth permissible if a fair test is to be made. This insures that a normal mature growth of the fungus in a vigorous state with characteristic spores will be pitted against the test substance. Some species of these genera scarcely begin to form their macrospores (''fuseaux'') in five days, which is the growth period set by the Reddish method.[2] To use an immature five-day culture of such a species will give an erroneously high value to the fungicide being tested because such young mycelium is more easily killed.

The glass bead method advocated by Klarmann is also open to criticism. Aside from a few ''water molds,'' parasitic on aquatic animals and plants, there are not many filamentous fungi which develop normally in a liquid medium. Although beads offer a platform upon which the fungous mat may rest, the hyphae cannot penetrate to obtain a firm hold. Shaking such relatively weak growth with the beads to form a suspension is a questionable practice. Hyphal walls and spore cells are ruptured, and the resultant suspension deteriorates before the real test begins. It is as if one were to take a geranium cutting, bruise it badly until the cells were broken and their contents oozing out, then pot it and expect a normal plant. In this connection, attention should be directed to a misinterpretation of my earlier study[5] by Burlingame and Reddish.[6] *At no time have I advocated the use of glass beads* in the preparation of either growth or suspension for testing.

In my experience the agar cup plate method has proved unsatisfactory for mycologic work, except to give confirmatory evidence of the results obtained by the test tube method, and for testing ointments or gels. The relation is negligible between a soft agar jelly and the horny, keratinous tissues of skin, nails, and hair which are the natural habitat of dermatophytic fungi. These pathogens do not penetrate into the deeper layers of the skin, nor into other tissues beneath it. Scars are not left from infection. If penetration must be included in a fungicidal test (which I am not ready to concede), then it should be studied through the use of fungi growing upon horn scrapings, nail clippings, hair, or skin. Although possible, this is scarcely practical because of the time usually required for such cultures to attain suitable growth—about six to eight weeks.

These fungi are strongly *aerobic*. (Even the fungi which infect internal organs, as the lungs, are harmful quite as much because of occlusion of the air spaces as because of actual attack upon the tissues.) *Temperature* is another important factor. Few species will grow well and many are killed if kept at blood temperature. (Our cultures are grown at 27° to 32° C. During hot weather they are placed in the refrigerator to retain their vitality.) Consequently, Taft's[4] delayed cup plate test is inconsistent with the nature of these organisms. The fungus is both overheated and smothered before one proceeds with the test.

The ideal method would be to test fungicides against actual conditions on patients. As this is not feasible, the next best way might seem to be the production and treatment of lesions on animals. This would be my method of choice except that it is limited to the species which are parasitic upon lower animals, hence does not include all the fungi pathogenic for human beings. However, we make considerable use of it wherever possible.

Details of the method I employ are outlined briefly below. The formula for Sabouraud's medium, the species of fungi used, and other explicit information are to be found in a previous report.[5] In addition to the two species mentioned, *Trichophyton interdigitale* Priestley and *Epidermophyton rubrum* Cast., two other dermatophytes are usually tested, *Achorion gypseum* Bodin and *Microsporum lanosum* Sab. Frequently *Aspergillus niger* Tieghem is included when information is desired as to the effect of the test substance on laboratory weeds.

I. Spore Suspension. Here is where the crux of the test really lies. A poorly prepared, deteriorated suspension is much easier to kill than one which contains the spores and mycelial elements of a normally vigorous, sporulating fungus.

In growing the cultures of skin fungi for test purposes, test tubes 18 by 140 mm. are used, with 8 c.c. of Sabouraud's glucose agar, slanted to leave a butt of about one-half inch, and planted while fresh so that growth will cover the entire surface. (Equivalent surface in flask cultures would, of course, do as well.) When mature growth has occurred, as described above, suspension is prepared by washing the surface with sterile, distilled water. For such species as Monilia and related forms, 10 c.c. per tube are used; for such cultures as Aspergillus and Penicillium, 12 c.c. per tube; for dermatophytic fungi, 8 c.c. per tube. The underlying medium should never be broken, but spores may sometimes need to be dislodged by gentle strokes of a bent wire or a loop.

Spores and mycelial fragments are prone to "clump" in groups, hence the suspension should be strained through sterile cloth—double layer, if gauze; single, if mull is used. This gives a homogeneous suspension with microspores (conidia), macrospores ("fuseaux"), and mycelial cells throughout the liquid. Because of these multiple elements in a fungous suspension, I have not found a counting chamber of value, and rely upon the growth obtained from a comparable area of a thrifty culture at a definite age to assure a comparable suspension; I believe this to be as nearly exact as one can reach in handling *living* materials. Control tubes are planted at the beginning and end of each dilution tested.

II. Test Substance. Dilution Series. There is no magic formula for a dilution series. The usual Food and Drug Administration method is readily applicable, except that planting should be made upon agar medium instead of liquid. In most cases, a preliminary, wide dilution series will set the limit for the final series of dilutions.

III. Exposure Intervals. We use one, five, ten minutes, and one hour exposure intervals. When *fungistatic* effect is to be studied, daily transplants are made for any desired period. We customarily allow three weeks.

IV. Incubation Period. For short life-cycle fungi, as Monilia, Cryptococcus, etc., final records may be taken seventy-two hours after planting.

For longer life-cycle species, as the dermatophytes, the Aspergilli, the Penicillia, etc., records should not be considered complete until at least one week after testing. Germination often may be *delayed* a few days, with a good growth appearing later.

For practical purposes, no other method yet devised is as satisfactory as this in vitro (test tube) method to give comparative criteria on liquid fungicidal agents. Its outstanding advantages are:

1. It uses only thrifty, mature cultures with their spores well developed as they are found in nature.

2. After the suspension has been properly prepared, this method is closely comparable to the well-known official method of testing germicides. (F.D.A., with or without the Shippen modification.) Adequate controls aid in correct interpretation of results.

3. It gives ample scope for individual choice of dilution series and time intervals.

4. Its results can be check-tested without much delay, provided a sufficient number of cultures was prepared in the original planting. In laboratories where much testing is carried on, cultures may be planted routinely at two- to three-week intervals so that they are available at all times in a suitable stage of development.

Bacteriologists, chemists, and general technicians should realize the necessity to consider the *mycologic nature* of species which they use in any fungicidal test. True data and fair comparisons are not possible unless this is done.

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THE CENTRIFUGE TECHNIQUE IN THE HETEROPHILE AGGLUTINATION TEST*

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CENTRIFUGATION offers a rapid and reliable method for agglutination tests. It was first established as a routine procedure by Schiff[1] in his monograph on blood grouping. The results of heterophile agglutination tests performed by the centrifuge method are here presented.

The test is done in Wassermann tubes, 1.3 cm. wide by 10.3 cm. long. Inactivated serum is diluted serially in steps of one-half and added to the tubes in a volume of 0.5 c.c. One-half cubic centimeter of a 2 per cent suspension of washed sheep cells which have been collected twenty-four hours previously is then added. The mixtures are centrifuged for five minutes at about 1,000 r.p.m., and are then shaken and read for agglutination. Table I compares 98 various sera and 5 sera from cases of infectious mononucleosis examined by the Paul-Bunnell and the centrifuge techniques.

TABLE I

POSITIVE IN DILUTION OF SERUM	WASSERMANN SERA		INFECTIOUS MONONUCLEOSIS	
	CENTRIFUGE METHOD*	PAUL-BUNNELL METHOD*	CENTRIFUGE METHOD	PAUL-BUNNELL METHOD
1:2	27	28		
1:4	23	26		
1:8	22	26		
1:16	13	6		
1:32	2	4		
1:64	5	2		
1:128	1			
1:256			1	1
1:512			1	2
1:1,024			1	1
1:2,048			2	1

*No agglutination was observed with either the centrifuge or the Paul-Bunnell methods in 5 and 6 sera, respectively.

It is seen from the table that with the centrifuge method there is a shift toward agglutination with higher dilutions of serum, indicating the greater sensitivity of this reaction. In no case, however, was a difference of more than one dilution observed. The rapidity with which the heterophile agglutination test can be completed by means of centrifugation gives it a distinct advantage over the Paul-Bunnell technique.

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THE APPLICATION OF AN ENDOSPORE STAIN TO BLOOD SMEARS FROM OPSONOPHAGOCYTIC TESTS*

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IN ORDER to obtain an accurate count of the bacteria phagocytized in an opsonophagocytic test, it is necessary to employ a stain which will cause the engulfed organisms to stand out distinctly within the cytoplasmic substance of the leucocytes. Recently, some difficulty was encountered in staining a *Brucella* strain of equine origin in blood smears made from opsonophagocytic tests. Wright's stain, although producing excellent results with the blood cells, did not color the bacteria intensely enough for an accurate count. Hastings' stain gave more deeply colored microorganisms, but the contrast between the bacteria and the phagocytes was not as striking as we wished for our tests. Since the organisms were gram-negative, the Gram stain was of no value. Other attempts failed to produce the desired differential staining until a modified form of the Schaeffer and Fulton[1] endospore stain was used.

Schaeffer and Fulton[1] simplified the endospore staining technique of Wirtz[2] by eliminating fixing in osmic acid, shortening the time of staining, and substituting aqueous safranine for dilute carbolfuchsin. The procedure as evolved by them consisted of the application of a 5 per cent aqueous malachite green solution to smears fixed by flaming. This stain was heated and allowed to stand for one-half minute. It was then washed off with top water after which 0.5 per cent aqueous safranine was added as decolorizer and counterstain. The spores were stained green and the cells red. By allowing the malachite green to act for a longer interval without heating and then applying safranine for a shorter period, we obtained a preparation in which the bacteria were stained green, the nuclei of the leucocytes a deep red, and the cytoplasm a light pink. There was no blending of the colors and phagocytized microorganisms stood out clearly as definite green bodies within the pink cytoplasm of the white blood cells.

The modified technique which gave the best results is as follows:

1. Make a smear from the test material by spreading the contents of a 2 mm. loop over an area of 4 sq. cm., air dry, and fix by flaming three times.

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2. Apply 5 per cent aqueous malachite green solution for 5 minutes.
3. Wash for 10 to 20 seconds with tap water.
4. Apply 0.5 per cent aqueous safranine solution for ten seconds.
5. Wash quickly with tap water, dry, and examine.

Heat fixation of a fairly heavy smear produced a preparation free from intact erythrocytes and at the same time retained a sufficient number of leucocytes within a limited area. Other differential stains were tried on similar slides, but in no case was the contrast between the bacteria and the white blood cells as marked as in the malachite green-safranine combination.

The technique is not applicable to all species of bacteria for some strains are unable to retain the malachite green stain in the presence of safranine. It was noted that staphylococci are stained red by this method whereas streptococci present a few green cells in a field occupied for the most part by red cocci. Some gram-negative bacteria are stained green and others become red under similar treatment.

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A NEW TUBE CLAMP*

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MOHR'S pinchcock is probably the one most widely used because it can be so quickly adjusted to the tubing, and because it is simple in design and low in cost. The only disadvantage of this pinchcock is that it can be adjusted to the tubing only by sliding it on from the end, and this sometimes involves disconnecting apparatus. To overcome this difficulty I have designed a pinchcock from two Mohr's pinchcocks, by cutting off from each, one jaw with its finger press, leaving about 16 mm. more jaw on one clamp than on the other. Holes are then cut for a bolt, the head of which is countersunk in what forms the inside surface of the jaw of the new clamp. The bolt is secured by a nut on the outside. This allows one finger press to be moved through a right angle, enabling a piece of rubber tubing to be taken into the clamp, or released from it, without having to slide the clamp over the end of the tubing.

The principle can be seen at a glance in the photographs of Fig. 1.

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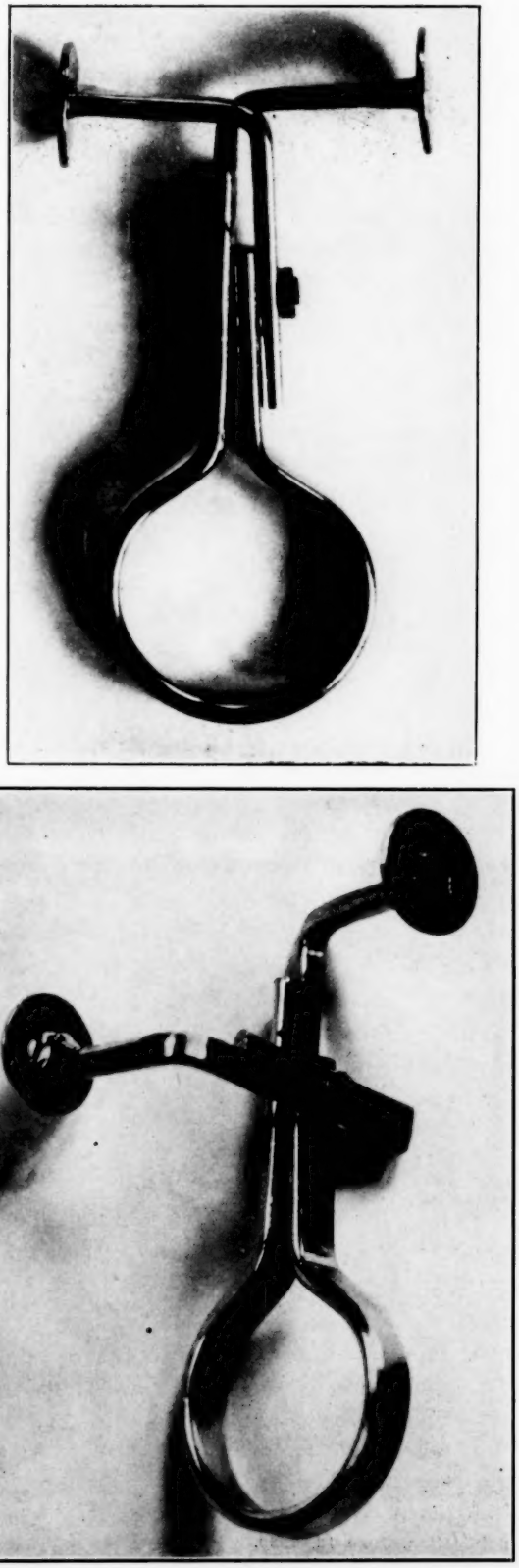

Fig. 1.—Modified Mohr's pinchcock. *Upper*, modification of ordinary type; *Lower*, finger press moved through a right angle.

A METHOD OF DESTROYING THE BLASTOCYSTS (BLASTOCYSTIS HOMINIS) IN FECAL WET SMEARS IN ORDER TO FACILITATE THE EXAMINATION FOR ENDAMOEBA HISTOLYTICA*

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IN ALMOST all texts dealing with the examination of feces for intestinal protozoa the reader is warned against blastocysts. Rightly so, because the beginner and even the laboratory worker with some experience frequently have trouble in differentiating the blastocysts from the cysts and rounded up trophozoites of *Endamoeba histolytica* and from some of the other intestinal protozoa. Undoubtedly not a few persons who harbor this innocent vegetable organism have been told that they have amoebic dysentery. We have seen this happen in a few instances.

There is a simple and practical way to prevent such mistakes. The blastocysts when placed in tap water or distilled water distend, rupture, and disintegrate as a result of endosmosis. The trophozoites of the intestinal protozoa are similarly destroyed, but the cysts withstand the decreased osmotic pressure and remain unchanged for many hours. Thus, if a fecal smear is made in water instead of the usual normal saline solution, blastocysts and protozoan trophozoites disappear, leaving a clear field for any cysts that may be present.

The practical significance of this should be obvious to those who labor with examinations of feces. With very few exceptions the identification of *E. histolytica* in clinical laboratories is made, or should be made, on the cysts. The search for cysts is usually made in a normal saline smear. Low magnification, for example 16 mm. objective and 7.5 ocular, has to be used in order to cover an adequate area within a reasonable time. With this magnification the blastocysts and the protozoan cysts, as well as the smaller trophozoites, usually appear so similar that they cannot with certainty be set apart. Whenever blastocysts are present, and they are present in the great majority of stools, it, therefore, becomes necessary to search with the 4 mm. objective. Few workers in clinical laboratories will have time for covering even a single smear with this high magnification. Furthermore, some forms of blastocysts resemble the protozoan cysts so closely that even with the 4 mm. objective each individual has to be scrutinized, all of which requires much time. A smear in tap water eliminates this nuisance. By the time the smear has been made and the preparation is ready to be examined the blastocysts have either disappeared completely or become so distended and lusterless that they readily can be differentiated from the shining refractile cysts.

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It should be mentioned that if a large particle of feces is used in making the aqueous smear, salts in the feces may dissolve in the water to such an extent as to make the smear almost isotonic and thus defeat the purpose of the smear. It is well, therefore, to pick up only the small particle of feces needed for the smear and avoid thick smears.

THE CAPILLARY HEMATOCRIT METHOD OF DETERMINING BLOOD CELL VOLUME*

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INTRODUCTION

IN UNDERTAKING an extensive survey of cellular and hemoglobin blood levels of University students and pre-school children, the problem of including cell volume determinations that did not necessitate securing the blood sample by venipuncture presented itself. A search of the literature disclosed several methods requiring small quantities of capillary blood. Since many methods have been criticized for their accuracy because they require dilution of the blood with an anticoagulant, an attempt was made to develop a technique which would not necessitate dilution of the sample. Several such methods have been described, among which are those of Daland,[1] Capps,[2] Epstein,[3] Rosahn,[4] Miller,[5] and Gram and Norgaard.[6] Of these, the technique and apparatus described by Rosahn seemed to be the most practical from the point of view of ease of manipulation and application to our work.

DESCRIPTION OF PIPETTES

Thick-walled glass tubing, possessing a uniform capillary bore, in sizes which varied in diameter of bore from 0.5 to 1.8 mm., were cut into 11 cm. lengths for fashioning into capillary pipettes. One end of the capillary pipettes was ground and fashioned to receive a rubber suction tube while the other end was ground to form a long bevel of 8 to 10 mm., the tip of the pipette being left at a diameter of 1.5 to 2 mm. The pipettes were closed by encircling them from end to end with rubber bands 3½ inches long and ¾ inch wide. Rubber bands narrower than ¾ inch would not stay in place while the pipettes were being centrifuged. Rubber bands alone were soon cut by the pipettes during centrifuging but rubber bands reinforced with automobile tire inner tube patches could be used in several hundred determinations. A short, wide-beveled pipette could not be properly closed with the reinforced rubber bands, a small but significant quantity of blood being lost into the small space formed between the rubber band and the tip of the pipette. The longer bevel described above resulted in effective closure of the pipette by eliminating

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this space. After centrifuging blood samples in these pipettes, the total blood columns and the red blood cell columns were measured on a millimeter ruler and the volumes of the red blood cells determined as per cent of the total volume of blood.

METHOD OF USE OF PIPETTES

A series of comparative studies were made to determine the accuracy of pipettes differing in diameter of bore. In one series of studies the pipettes were filled with venous blood prepared for cell volume determinations by the Wintrobe method,[7, 8] determinations using both the Wintrobe tubes and capillary tubes being carried out simultaneously on the same sample of blood. In another series of studies the capillary pipettes were filled with capillary blood for comparison with Wintrobe determinations of cell volume on venous blood of the same individual. Pipettes with bores of 0.5, 0.6, 0.8, 1.0, 1.2, 1.3, and 1.8 mm. in diameter were used in these studies. It was found that the pipettes of smallest bore frequently showed cell volumes 0.5 to 1 per cent lower than those obtained with the larger pipettes. Determinations made with the three largest pipettes varied 0.5 per cent higher or lower than determinations of the cell volume on the venous blood of the same individual by the Wintrobe method. The pipettes of 0.8 and 1.0 mm. bores were found to be accurate and were adopted for use. In this series of studies and in all subsequent studies, the pipettes were centrifuged until a constant cell volume was obtained, fifteen minutes being required for the angle centrifuge (3,800 r.p.m.) and thirty minutes for the horizontal type of centrifuge (3,200 r.p.m.).

Blood Not Mixed With Anticoagulant.—Though fairly good packing of the red blood cells was secured with clean pipettes centrifuged immediately after being filled with capillary blood, it was clearly evident that coating the pipettes with a small quantity of anticoagulant which would not significantly alter the results by dilution of the blood sample would be desirable. As sodium citrate gave the best initial results, further experiments were conducted to learn its most effective concentration and method of use. Saturated solutions were diluted to make one-fourth, one-third, one-half, two-thirds, and three-fourths saturated solutions. The higher concentrations produced a significant amount of shrinkage while the one-fourth saturated solution did not give consistent results. The one-third saturated solution was consistently effective and, when properly used, did not produce significant changes through shrinkage or dilution. In practice, the one-third saturated solution was drawn into the pipette, the excess expelled, and the pipette partially dried by blowing through it. If the pipettes were centrifuged immediately after being filled, the cells packed completely. In this series of studies it was learned that fungi grew rapidly in the one-third saturated citrate solutions and that when solutions containing fungi were used packing of the cells could not be secured. They did not grow, however, in saturated citrate solutions, and the practice of making up the dilute solutions just before they were to be used was therefore adopted.

Though the method of using sodium citrate, as described above, yielded consistently accurate results, other anticoagulants were also tried. The ox-

alates and oxalate mixtures did not always yield accurate packing of the cells. If they acted effectively as anticoagulants, they usually produced significant shrinkage of the cells. Heparin used as a 1 per cent solution in the same manner as the one-third saturated sodium citrate solution proved effective, yielding the same results as the citrate solution.

None of the anticoagulants used for wetting the pipettes before filling them with capillary blood in the manner described above was effective for more than a few minutes after the pipettes had been filled, poor packing of the cells resulting if the pipettes were allowed to stand for a short time before being centrifuged. Wetting the pipettes with the anticoagulants, including heparin, and drying them completely before filling them with blood was also ineffective. In all cases the failure of the cells to pack after delayed centrifuging was due to the formation of coagula in the center of the pipette and in some limited areas along the side of the pipette, pointing to incomplete mixture of the blood and anticoagulants as the direct cause of failure of the cells to pack.

Blood Samples Mixed With Anticoagulants.—Since mixing of the blood and minute quantities of dry anticoagulants could not be effected in the pipettes and delayed centrifuging of the blood samples was sometimes necessary, mixing of the blood and dry anticoagulants before filling the pipettes was attempted as a means of obtaining accurate results when centrifuging was delayed. Guest and Siller,[9] Kato,[10] Smith,[11] Andresen,[12] and Rogatz[13] have described methods of mixing small quantities of blood and anticoagulants. The method of using a depression slide for receiving the blood and mixing it with dry anticoagulants, as described by Kato, appeared to be the best suited to our work. The depression (15 mm. in diameter and 3 mm. deep) of a slide was coated with the anticoagulant, dried, and an unmeasured quantity of blood, added directly from a finger puncture, was thoroughly mixed with the anticoagulant. A 2 per cent solution of a mixture of ammonium and potassium oxalate (0.8 gm. potassium oxalate and 1.2 gm. ammonium oxalate in 100 c.c. of distilled water) recommended by Kato[10] and Heller and Paul[14] was used. Like Andresen,[12] we found this mixture to be unreliable, even when centrifuging of the blood sample was carried out immediately. Either coagulation was not completely prevented or shrinkage occurred. Changing the concentration of the mixture and the amount placed on the depression slide, before drying and before the blood was added, did not correct the condition to the extent that the method could be considered reliable. Sodium oxalate, potassium oxalate, and sodium citrate were tried in the usually recommended concentrations, as well as in various modified concentrations, but with no better success than with the oxalate mixtures. Though fairly satisfactory packing of the red blood cells could be obtained if centrifuging was carried out immediately, satisfactory packing with delayed centrifuging could be secured only when shrinkage occurred. Since the quantity of blood added to the depression slide was not constant, even though the depression appeared filled, it was impossible to secure a precise proportion of the anticoagulants to blood. Hence shrinkage of different degrees for different samplings of blood occurred, and corrections for shrinkage could not be made

with certainty. Obviously this method of mixing inorganic anticoagulants and blood for cell volume studies lacks necessary accuracy.

A modification of the depression slide method by placing definite quantities of anticoagulant and of blood in the depression was more successful than the method already described but did not always yield accurate results. Also the method involving the use of definite quantities of blood and anticoagulant became very complicated as to technique and apparatus required for maintaining accuracy. Since, as Andresen[12] also has pointed out, precise methods employing inorganic anticoagulants could not always be relied upon for consistently accurate results, heparin was used as the anticoagulant. It was used as a 1 per cent solution as recommended by Rosahn,[4] Andresen,[12] and others. A drop of the solution was placed in the depression of the slide, dried, and blood added directly from a finger puncture until the depression was three-fourths to practically completely filled. This was found to prevent coagulation for long periods of time. Pipettes filled with blood from the depression slide immediately after mixing with the dried heparin showed complete and accurate packing of the blood cells after standing for 1½ hours before being centrifuged, as shown in Table I. Though good packing of the red

TABLE I

COMPARATIVE CELL VOLUME DETERMINATIONS

SUBJECT	CELL VOLUME IN PER CENT OF HEPARINIZED CAPILLARY BLOOD COLLECTED IN DEPRESSION SLIDE AND CENTRIFUGED IN CAPILLARY PIPETTES								CELL VOLUME IN PER CENT OF OXALATED VENOUS BLOOD CENTRIFUGED IN WINTROBE TUBES	
	PERIOD OF TIME PIPETTES ALLOWED TO STAND BEFORE CENTRIFUGING									
	NONE		15 MIN.	30 MIN.	45 MIN.	1 HOUR	1¼ HOURS	1½ HOURS		
I	40.2		40.1	39.9	40.7	40.7	40.2		40.5	
II	48.4	47.3							47.0	47.0
	47.2	47.3	46.0	46.1	46.1	46.0	45.9	46.6	47.0	47.0
III	41.2		41.8	40.7	41.3	41.0	41.2	41.1	41.0	41.0
									41.0	41.5

blood cells can be secured after centrifuging is delayed for a period of an hour or more, it is advisable to centrifuge as soon as possible to insure accuracy, particularly if an angle centrifuge is used, for a few cells tend to adhere to the portion of the wall of the pipettes against which the centrifugal force is directed.

Only one precaution is necessary for the last-described technique, that the proportion of heparin to blood must not be too great. If a small drop (approximately 3.0 c. mm. for these studies) of a 1 per cent solution of heparin is used, only enough to leave a visible deposit at the edge of the depression, a precise quantity of blood need not be added. If this general rule is followed, changes in volume of the cells will not occur.

SUMMARY

1. The capillary pipette method of determining cell volume of capillary blood drawn directly into the pipette without previous mixing with an anticoagulant is a satisfactory method if centrifuging is carried out immediately.

Wetting the pipette with anticoagulants and blowing out the excess fluid aids in securing consistently accurate results. Inorganic anticoagulants used in concentrations ordinarily mixed with blood are, as a rule, not an aid to accuracy, stronger solutions being required. A one-third saturated sodium citrate solution is constantly effective when centrifuging is not delayed. A 1 per cent solution of heparin is also effective when used in a similar manner.

2. Mixing of dried inorganic anticoagulants and small but unmeasured quantities of capillary blood in depression slides before filling the pipettes for delayed centrifuging is subject to too great an error for practical use.

3. Only heparin thoroughly mixed with small and approximate quantities of capillary blood before the pipettes are filled permits delayed centrifuging of the blood sample with complete and accurate packing of the blood cells.

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DEPARTMENT OF REVIEWS AND ABSTRACTS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

PHOSPHATASE in Chronic Arthritis, Steinberg, C. L., and Suter, L. C. Arch. Int. Med. **64:** 483, 1939.

Determinations of the phosphatase content of the serum were made for 44 patients with atrophic arthritis, 8 patients with hypertrophic arthritis, 5 patients with mixed atrophic and hypertrophic arthritis, and 5 patients with osteitis deformans. This same study was made of 6 healthy persons. In the only case of arthritis in which the value for serum phosphatase was above 4.2 units, it was later proved that malignant tumor of the prostate gland was present, with secondary involvement of bone and lung tissue. An increase of serum phosphatase in this type of malignant disease has been described by Gutman and his co-workers. Determination of the phosphatase content of the serum in cases of chronic atrophic and hypertrophic arthritis is, therefore, important in the differential diagnosis. A normal value for serum phosphatase is characteristic of chronic atrophic or hypertrophic arthritis; an abnormal value suggests the possibility of a complicating condition or an erroneous diagnosis of this condition. Determinations of the phosphatase content of the serum should be made a routine procedure in the study of diseases of the bones and joints.

ENDOCARDITIS, Streptococcus Viridans, Middleton, W. S., and Burke, M. Am. J. M. Sc. **198:** 301, 1939.

Certain details in this clinicopathologic analysis of 88 patients with *Streptococcus viridans* endocarditis lenta justify especial emphasis:

1. Further evidence is adduced to support the thesis of a close relationship between congenital and rheumatic lesions of the heart and endocarditis lenta.

2. Acute upper respiratory infections, rheumatic fever, infected abortion, dental extraction, and massage for nonspecific prostatitis, apparently served as precipitating factors in the development of certain cases of this condition.

3. Contrary to the accepted opinion, congestive heart failure may attend or mask this condition.

4. The clinical manifestations and course of this affection are notoriously varied and inconstant. After the cardiac changes incident thereto, particular attention has been directed to its toxic and its embolic features. Splenic and renal changes, including embolism, were very frequent. Mycotic aneurysms offered serious diagnostic problems. Cerebral accidents were not infrequent. Occasionally a mycotic aneurysm of a cerebral vessel may explain certain neurologic phenomena of this condition. Again the clinical picture may suggest thyrotoxicosis, and the unexplained elevation of the basal metabolic rate may add to the diagnostic confusion.

5. This study offers material support to the importance of the diagnostic triad, i.e., petechiae, splenomegaly, and a positive blood culture for the *Streptococcus viridans*. Given the background of a congenital or a rheumatic heart lesion and a remittent fever, this triad offers the logical direction of attack.

6. The prognosis of *Streptococcus viridans* endocarditis lenta is very grave. Only inferential evidence of the pace of the decline is offered by the circulatory, renal, embolic, toxic, constitutional, and hematologic reactions. Although remissions of varying durations and degrees are the rule, certain patients undergo a rapidly progressive decline. Attention has been directed to the ominous significance of the euphoria that attends late remissions.

7. This group included one instance of healed endocarditis lenta. The clinical activity apparently occurred at a period removed from the hospitalization. All therapy in the remaining number (87) was unavailing.

PNEUMONIA, Treatment of, With Type-Specific Immune Rabbit Serum, Callomon, V. B. Am. J. M. Sc. **198**: 349, 1939.

A report is made on the treatment of 45 cases of pneumococcus pneumonia with homologous type-specific immune rabbit serum. Two deaths occurred in this series.

Premature recovery by crisis or rapid lysis ensued in 36 cases.

No serious serum reactions were observed.

BLOOD PLATELETS, in Pernicious Anemia, Paddock, F. K., and Smith, K. E. Am. J. M. Sc. **198**: 372, 1939.

In marked pernicious anemia a definite thrombocytopenia is rarely lacking. This is borne out not only by the authors' figures of 90 counts, but also by those collected from the literature.

The platelets increase in number parallel to the red blood cell count on the institution of specific therapy.

This diminution of platelets is apparently of no value in differentiating this type of anemia from others with a macrocytosis.

PNEUMONIA, Prognostic Significance of Eosinophiles in, Bracken, M. M. Am. J. M. Sc. **198**: 386, 1939.

Eosinophiles are entirely absent from the blood early in the disease in severe pneumonia, but may be present early in milder cases.

The appearance of eosinophiles in the blood of pneumonia patients treated with hydroxyethylapocupreine or not specifically treated is an index of recovery, and may precede clinical improvement by one to several days.

Eosinophiles were not found in the blood of patients dying of uncomplicated pneumonia.

The reappearance of eosinophiles during the course of pneumonia, while in itself a favorable sign, does not exclude the possibility of a serious and even fatal complication developing later.

MONONUCLEOSIS, Infectious, Mitotic Leukoblasts in the Peripheral Blood in, Bowcock, H. Am. J. M. Sc. **198**: 284, 1939.

In infectious mononucleosis, mitotic leucoblasts and other unusual cell forms may appear in the peripheral blood near the peak rise of the leucocyte count. The occurrence of such immature and unusual cell forms is emphasized in order that confusion may be avoided in the diagnosis of this relatively benign illness which often resembles acute leucemia in many respects.

CANCER, Temperature Factors in, and Embryonal Cell Growth, Smith, L. W., and Fay, T. J. A. M. A. **113**: 653, 1939.

Relatively low body surface temperatures normally exist in the segments concerned with the extremities (from 88° to 90° F.) and the breast segment (fifth thoracic) tends to maintain a higher surface temperature than the adjacent segments of the trunk. There is relative infrequency of primary or metastatic carcinoma developing in those parts of the body enjoying reduced temperatures, as compared to the organs and portions of the body where optimal high temperatures are found.

Certain clinical, pathologic, and biologic evidence indicates that young, undifferentiated cell growth and activity require an optimal temperature and that "critical" temperature levels exist below which these cells become inactive or undergo degenerative changes.

Local and general measures of "refrigeration" applied to patients suffering from hopeless metastatic carcinoma have been discussed, and the effects of these reduced temperatures on cell growth and activity, shown by serial biopsy and tissue cultures, have been indicated.

A "critical" level of around 95° F. has been noted below which undifferentiated cell growth, as exemplified by carcinoma pathologically and by the development of chick embryos normally, is arrested. Marked degenerative changes have been noted after seventy-two hours in biopsy specimens from carcinomatous tissues when continuously subjected to temperatures of 90° F. or below. Our observations have shown that normal cellular tissue is capable of withstanding 40° F. for prolonged periods of time without evidence of degenerative changes and normal reparative processes occur in carcinomatous areas maintained at this level. Tissue culture studies have confirmed these clinical observations.

Relief of pain encountered in terminal states of metastatic carcinoma has promptly followed "refrigeration" of the area involved or induced states of "hibernation" in which the patient has been maintained at levels of rectal temperature between 81° and 90° F. for periods as long as from one to five days.

Our observations suggest that the application of subcritical temperatures, through methods of local and general "refrigeration" may offer a valuable therapeutic adjunct to our present method of treatment of undifferentiated cell growth, particularly of carcinoma. Its practical clinical value and possibilities must await wider experience and more extensive and intensive observations.

HORMONES, Female, Summary of Modern Views on, and Their Scope in Therapy, Cunningham, A. J. M. J. Australia 2: 58, 1939.

A few brief facts showing the importance of the recognition of vitamin deficiency in pregnancy are the following:

1. Deficiency of vitamin A produces, among other things, alterations in the visual purple, giving rise to one of the earliest and most frequent of its symptoms, so-called night blindness or nyctalopia.

2. A not uncommon and distressing feature of pregnancy is polyneuritis; this in many cases is due to a vitamin B_1 deficiency.

3. Deficiency of vitamin C may give rise to dental caries of pregnancy and bleeding gums, and possibly also to disorders of the gastrointestinal function.

4. Deficiency of vitamin D tends to produce latent rickets in the fetus and also tetany in the mother.

5. The relationship of vitamin E deficiency and habitual abortion has recently been established.

These few brief facts show the interesting possibilities of vitamin therapy in pregnancy. The future will undoubtedly see further advances in vitamin research.

GRANULOCYTOPENIA, in Sulfapyridine Therapy, Dolgopol, V. B., and Hobart, H. M. J. A. M. A. 113: 11, 1939.

A review of the recent literature shows that 3 persons with granulocytopenia and a number of persons with leucopenia were observed in the course of sulfapyridine therapy.

Two new cases of granulocytopenia and 2 cases of leucopenia are reported in children with pertussis and pneumonia treated with sulfapyridine.

In the fatal case of granulocytopenia reported here, the patient lived nine days following a complete disappearance of polymorphonuclear cells. Minimal tonsillar lesions appeared two days before the patient died of lobar pneumonia. The pneumonic exudate was free of polymorphonuclear cells. The bone marrow was immature. Aplastic anemia developed in the course of granulocytopenia, but the megakaryocytes remained intact.

In the second case of granulocytopenia immature cells of the myeloid group appeared in the circulating blood with the beginning of recovery and were gradually replaced by more mature forms.

All 5 patients with granulocytopenia received large total amounts of sulfapyridine during the course of treatment.

In at least 2 cases of granulocytopenia sulfapyridine was apparently the only cause of the condition. In the other 3 cases a coincident damage to the bone marrow by the original infection or by a previous infection may be considered to be a contributory factor in the development of granulocytopenia.

In 2 cases granulocytopenia developed after the discontinuance of the sulfapyridine therapy.

A high total intake of sulfapyridine may cause granulocytopenia.

The damage to the bone marrow consists in depression of maturation of myeloid cells. The erythropoiesis may also be occasionally disturbed.

Blood counts should be made twice a week during sulfapyridine therapy and continued for two weeks after the withdrawal of the drug.

LEUCOCYTES, Total Differential and Absolute Counts and Sedimentation Rates in Healthy Children 4-7 Years of Age, Osgood, E. E., Baker, R. L., Brownlee, I. E., Osgood, M. W., Ellis, D. M., and Cohen, W. Am. J. Dis. Child. **58:** 61, 1939.

There are no significant age or sex differences in the total, differential, or absolute leucocyte counts, or in the sedimentation rates for children 4 to 7 years of age.

STATISTICAL AND SMOOTHED MEANS AND RANGES FOR TOTAL, DIFFERENTIAL, AND ABSOLUTE LEUCOCYTE COUNTS AND SEDIMENTATION RATES FOR CHILDREN 4 TO 7 YEARS OF AGE

		STATISTICAL MEAN		PROBABLE ERROR DISTRIBUTION		SMOOTHED MEAN	95% RANGE
Leucocytes	per c.mm.	10,419	176	1,632	84	10,400	5,500-15,500
Neutrophiles	per cent	37.3	0.9	7.9	0.4	38*	16-60
Lobocytes	per c.mm.	2,959	124	Skew		4,000	1,500-7,500
Neutrophiles	per cent	----------		----------		3*	0-10*
Rhabdocytes	per c.mm.	----------		----------		250*	0-1,000*
Eosinophiles	per cent	----------		----------		3*	0-8*
Lobocytes	per c.mm.	----------		----------		300	0-800
Basophiles	per cent	----------		----------		0.5*	0-2*
Lobocytes	per c.mm.	----------		----------		50†	0-200†
Lymphocytes	per cent	48.9	1.0	10.1	0.5	48*	20-70*
	per c.mm.	5,020	140	Skew		5,000	1,500-8,500
Monocytes	per cent	----------		----------		3*	0-7*
	per c.mm.	----------		----------		300†	0-800†
Disintegrated cells	per cent	----------		----------		5*	0-12*
	per c.mm.	----------		----------		400*	0-1,200*
Sedimentation rate	15 min.	----------		Skew		2†	0.5-5.0†
	45 min.	----------		Skew		10†	1.0-30.0†

*Applies also to 8- to 14-year-old boys and girls.
†Applies also to persons 8 years of age or older.

TRANSFUSIONS, Blood, The Use of Heparin in, Sappington, S. W. J. A. M. A. **113:** 22, 1939.

A stable, potent, pure heparin was used as the anticoagulant in 40 blood transfusions and appeared to be without toxic or other undesirable effects on donor or patient. It was used in two ways: first, in vitro to heparinize the drawn blood from the donor; second, in vivo to heparinize the donor by intravenous injection. The first corresponds to transfusion of citrated blood and differs from it only in the anticoagulant. The second method is simpler in that the heparinized blood is withdrawn from the donor and introduced into the patient without any treatment outside the body. Seventeen patients were given transfusions by the first method, and 23 by the second, all with 500 c.c., since a larger amount of blood was thought a better test of the procedure. These were all done by a gravity method paralleling the citrate technique in order that comparison of the two anticoagulants could better be made.

All 40 transfusions were successful in that the entire amount of blood was transfused and the donors and patients suffered no ill effects. But it was found that for a successful

uninterrupted transfusion a larger needle was necessary for introduction into the patient than in the citrate method, a 16 gauge needle as compared with an 18 gauge commonly used in citrate transfusion. If a 500 c.c. transfusion is desirable, this has a disadvantage in that the patient's veins may be difficult to enter with such a large needle. If a small transfusion is sufficient, up to 350 or even 400 c.c. may be given without difficulty with an 18 gauge needle. Or the operation of giving 500 c.c. can be accomplished by giving as much blood as will flow through the small needle and then completing the transfusion by making another venipuncture with a fresh 18 gauge needle. The two punctures caused little or no discomfort to the patient. This hindrance to the heparin transfusion is caused by clotting within the smaller needle, even though the same heparinized blood may be kept days without clotting. In one instance blood was banked twenty-four hours and in three instances forty-eight hours and then transfused successfully. Reactions are negligible, as they are now in transfusions with citrated blood. Whether the heparin method can displace the well established citrate method remains to be seen. It would seem to offer two advantages. The heparinization of the donor makes the procedure more simple and almost a one-man job. The therapeutic value of heparin may be of advantage in post-operative transfusions.

DIPHTHERIA, Potassium Tellurite in the Diagnosis of, Tomlin, E. Brit. M. J., June 24, 1939, p. 1273.

The technique of this suggested clinical test for diphtheria is simple.

A negative result is of value in that it supposes with great accuracy (100 per cent in this series, 92 per cent in that of Dr. Manzullo) that the disease is not diphtheria.

It is unlikely that any case of diphtheria would be missed through reliance being placed on the test, if the technique were not at fault.

Such a high percentage of false positive results may be obtained that no definite diagnosis of diphtheria should be made on a positive result.

It is possible theoretically, and in view of the discrepancy between these results and those of Manzullo, that the proportion of false positive results will vary from place to place and from time to time.

This test can in no way take the place of the clinical and bacteriologic methods of diagnosis already in use.

TUBERCLE BACILLI, Preservation of, Cohn, M. L. Am. Rev. Tuberc. 40: 99, 1939.

Desiccated human (virulent and avirulent), bovine (virulent), and avian (virulent) tubercle bacilli almost completely retain viability for three years at refrigerator temperature, while natural cultures survive under the same condition of temperature only about six months to one year (occasionally two years). The loss of viability of the desiccated cultures of these same strains of tubercle bacilli is much more rapid at incubator temperature, being almost complete at six months to one year; while at room temperature, they survive a little better than at incubator temperature.

The loss of viability of tubercle bacilli is primarily a function of the temperature at which they are stored, regardless of whether they are desiccated or natural. The rate of loss of viability of cultures of tubercle bacilli is greater in the presence of oxygen, less in the presence of air, and least in nitrogen. Desiccation, although a minor factor, aids in maintaining the viability of tubercle bacilli. Desiccation can remove as much as 70 to 75 per cent of the water (and volatile materials) from the cultures of tubercle bacilli without appreciable detrimental effect on the viability. Aside from the preservation of well-grown broth cultures of mammalian tubercle bacilli (some of the bacilli remaining viable for over twelve years, as described by us in 1933), cultures of strains of mammalian and avian tubercle bacilli can be preserved in viable form for over three years when desiccated and sealed in air or nitrogen and maintained at refrigerator temperature. The latter method possesses advantages over the former in that the specimens occupy little space and are readily transportable.